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13. ABSTRACT ***(Maximum 200 Words)***

Understanding the biological mechanisms that control cell growth and cell death is a mayor goal in cancer research since it could allow the design of anti-neoplasic therapies to specifically eliminate tumor cells. The effectiveness of radiotherapy and chemotherapy relies in part in their ability to induce a genetic program of cell destruction know as apoptosis. Unfortunately, at present this conventional therapies induce cell death of both normal and cancer cells resulting in undesirable toxic effects. Furthermore, the signaling pathways that lead to apoptosis are often subverted in cancer cells resulting in resistance to radiation or chemotherapy. One mechanism by which cancer cells become resistant to radiation is by disrupting the sphigomyelin pathway that comprises a series of biochemical reactions generating lipid molecules called sphingolipids that are involved in controlling various cellular functions. The sphingolipid metabolites ceramide, sphingosine and sphingosine-1-phosphate (SPP) have recently emerged as a new class messengers that regulate cell growth and apoptosis. While intracellular accumulation of sphingosine or ceramide induces cell death, SPP promotes cell survival. Therefore, our laboratory has proposed that a dynamic balance in the level of intracellular sphingolipids serves as a signal to determine whether a cell survives or dies. In support of this hypothesis it has been found that the sensitivity of specific prostate cancer cell lines to radiation correlates with intracellular ceramide levels and with the activity of sphingosine kinase (SPHK), the enzyme that catalyzes the formation of SPP after adding a phosphate group to sphingosine. I propose to further investigate the role of sphingolipids in radiation-induced apoptosis in prostate cancer. First, prostate cancer cell lines with different susceptibility to radiation will be used to determine the mass levels of ceramide, sphingosine and SPP at different times after apoptotic stimulation. Elevated intracellular levels of ceramide and sphingosine are expected to be found in association with high radiosensitivity. Second, the levels of expression of SPHK will be experimentally enhanced or inhibited in prostate cancer cells and the susceptibility to apoptosis will be studied. Since SPHK produces SPP, decrease sensitivity to radiation is expected if high levels of this kinase are achieved. Finally, isolation of different forms of human SPHK will be attempted from known DNA sequences with similarity to murine SPHK. Achievement of this goal could facilitate characterization of role of sphingolipid metabolites in human cancer.
In summary, studying the role of sphingolipids in the modulation of susceptibility of prostate cancer cells to apoptotic may provide the basis for molecular strategies that improve existing anti-neoplasic therapies.

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INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer deaths in men (1). Radiation therapy that causes growth inhibition and apoptosis is often used for treatment of both primary and metastatic prostate cancer. However, despite using high doses of radiation, about 20-25% of prostate cancer patients with non-invasive disease (stages T_1-T_2) relapse. A major reason for failure to eradicate local disease is the intrinsic radioresistance of the tumors. Ionizing radiation mediates cell death, in part, through chromosomal damage and also by induction of apoptosis. Although apoptosis appears to be less prevalent than clonogenic cell death, one mechanism by which cancer cells become resistant to radiation or chemotherapy is by disruption of pathways leading to apoptosis.

Abundant evidence suggests that the sphingolipid metabolite, ceramide, is a critical component of ionizing radiation-induced apoptosis (2-5). This apoptotic pathway is initiated by hydrolysis of sphingomyelin, a membrane lipid, due to activation of sphingomyelin-specific forms of phospholipase C, termed sphingomyelinases (SMases), to generate ceramide. Ceramide, in turn can activate several pathways important for induction of apoptosis (reviewed in (6, 7)). Both neutral and acidic SMases, distinguishable by their pH optima, have been reported to be involved in the induction of apoptosis following ionizing radiation (reviewed in (8, 9)). Acidic SMase may play an essential role in radiation-induced apoptosis, as lymphocytes from individuals with Niemann-Pick disease, who have an inherited deficiency of acidic SMase, and acidic SMase deficient mice, do not generate ceramide or have defective apoptotic responses to ionizing radiation (4). These deficits are reversible upon restoration of acidic SMase activity, further substantiating the obligatory role for ceramide generation in these apoptotic responses. However, ionizing radiation-triggered apoptosis of sensitive, but not resistant human myeloid leukemic cell lines, correlated with sphingomyelin hydrolysis and ceramide generation through activation of neutral, but not acidic, SMase (10). Similarly, loss of ceramide production from a neutral SMase confers resistance to radiation-induced apoptosis in lymphocytes (5). Moreover, depletion of glutathione, an endogenous inhibitor of neutral SMase (11), may also contribute to its activation, since glutathione depletion occurs in a variety of cells during radiation-induced apoptosis (12, 13). In addition, it has been suggested that *de novo* synthesis of ceramide as a result of increased ceramide synthase activity may also be involved in apoptosis (14), particularly in radiation-insensitive LNCaP prostate cancer cells induced to die by the phorbol ester TPA (15). LNCaP cells express androgen receptor, and their growth is increased by androgen. However, because they do not undergo apoptosis after androgen withdrawal, these cells can be used as an *in vitro* model to study strategies for treating prostate cancers that are resistant to androgen ablation. LNCaP cells are highly resistant to apoptosis induced by γ-irradiation, although somewhat sensitive to the death-inducing effects of TNF-α. Recently, we have shown that TNF-α sensitizes LNCaP cells to γ-irradiation-induced apoptosis by elevating ceramide levels (16). Moreover, exogenous C_2-ceramide also sensitized LNCaP cells to

irradiation, lending further support to the notion that ceramide generation might be important for radiation-induced apoptosis in human prostate cancer.

One metabolite of ceramide, sphingosine, formed by ceramidase, has also been implicated in cell growth arrest and apoptosis. Sphingosine is rapidly produced during TNF-α-mediated apoptosis in human neutrophils (17) and cardiac myocytes (18). Recently, it has been shown that sphingosine and other long chain sphingoid bases induce apoptosis in hepatoma cells by activation of caspase-3-like proteases (19). Moreover, in androgen-independent human prostatic carcinoma DU-145 cells which express bcl-X_L, sphingosine, but not its metabolites, induced apoptosis by down-regulation of bcl-X_L, independently of protein kinase C (PKC) inhibition (20). In contrast to the growth suppressing and pro-apoptotic roles of ceramide and sphingosine, sphingosine-1-phosphate (SPP), formed from sphingosine by activation of a unique kinase (21), has been implicated in cellular proliferation and survival induced by PDGF, serum, NGF, and vitamin D3, and protects cells from apoptosis resulting from elevations of ceramide (22-26). In this report, we examined the role of ceramide, sphingosine, and sphingosine kinase in sensitization of radio-resistant LNCaP prostate cells to γ-irradiation-induced apoptosis.

BODY

SPHINGOSINE ENHANCES APOPTOSIS OF RADIATION RESISTANT PROSTATE CANCER CELLS

Sphingomyelinase treatment sensitizes LNCaP cells to γ-irradiation induced apoptosis

Previously, we have shown that treatment with C_2-ceramide synergizes with γ-irradiation to induce cell death in LNCaP cells (16). In agreement, we have found that increasing endogenous long-chain ceramide levels in LNCaP cells by pretreatment with SMase induces apoptosis and sensitizes the cells to a dose of γ-irradiation (8 Gy) sufficient to trigger apoptosis of TSU-Pr1, but not of LNCaP cells (Fig 1). Apoptosis increased in a time-dependent manner and at least 48 h were required for significant cell death. To confirm the induction of apoptosis, we also examined DNA fragmentation and nuclear condensation by staining with the DNA-specific fluorochrome bisbenzimide (Hoechst 33258). Substantially more DNA ladder formation and fragmented nuclei were seen after 72 h than 48 h (data not shown).

To examine whether there was a correlation between ceramide levels and radiation sensitivity, we measured changes in ceramide levels after irradiation of these two cell lines. Exposure of radiation-sensitive TSU-Pr1 cells to 8 Gy irradiation led to acute, but small, increases

in ceramide levels within 30 min, followed by a sustained elevation in ceramide 12 h after irradiation that reached a 3-fold increase by 48 h (Fig. 2A). This generation of ceramide preceded the appearance of nuclear fragmentation which was evident only after 48 h (Fig.1). In contrast, ceramide levels did not change in radio-resistant LNCaP cells (Fig. 2A). In addition, irradiation induced a rapid and sustained decrease in sphingosine kinase activity in TSU-Prlcells but not in LNCaP cells (Fig. 2B). A surge in sphingosine levels was detected in TSU-Pr1 cells in correspondence with the inhibition of sphingosine kinase (Fig. 2C). SPP levels in the radiosensitive TSU-Pr1 cells were below the detection limit (<0.01 pmol/nmol phospholipid) and thus we were unable to detect any increases after irradiation. Interestingly, levels of SPP in TSU-Pr1 cells were much lower than the SPP levels in LNCaP cells (0.12 pmol/nmol phospholipid), which are more resistant to γ-radiation. Thus, there appears to be a reciprocal relationship between ceramide/sphingosine and sphingosine kinase in radiation-sensitive prostate cancer cells.

Sphingosine generation in apoptosis-sensitized LNCaP cells

Because it has been suggested that sphingosine, a breakdown product of ceramide, might also be a mediator of programmed cell death (19), it was of interest to determine whether sphingosine could be involved in the sensitization of LNCaP cells to apoptosis induced by irradiation. Treatment with bacterial SMase, which markedly sensitizes these cells to radiation (Fig. 1), as expected, induced a rapid 4 to 5-fold elevation in ceramide levels, which remained elevated at 24 h and declined thereafter (Fig. 3A). Interestingly, SMase treatment also increased intracellular sphingosine after 24 h (Fig. 3B). It is likely that this increase in sphingosine results from degradation of ceramide as the ceramide increase preceded that of sphingosine. It should be pointed out that the elevation of these two sphingolipid metabolites precedes the onset of apoptosis (Fig. 1). As previously shown (16), we found that TNF-α also sensitized LNCaP cells to irradiation with a concomitant increase in ceramide levels (Fig. 4A and 5A). Although irradiation alone did not increase sphingosine levels in LNCaP cells, irradiation together with TNF-α induced significantly higher sphingosine levels than TNF-α alone (Fig. 4B). The increase in sphingosine was detected 24 h after treatment, coinciding with elevation in ceramide levels and preceding nuclear fragmentation that was evident 48 h after treatment (16). In LNCaP cells sensitized to die by irradiation and TNF-α, there was a marked decrease in SPP levels 48 h after treatment, whereas no significant changes in SPP levels were detected in cells treated with γ-irradiation alone (Fig. 4C). It should be noted that SPP levels in LNCaP cells (0.12 ± 0.01 pmol/nmol phospholipid) are greater than in other normal and cancer cells including HL60 (0.01 ± 0.001 pmol/nmol phospholipid), PC12 pheochromocytoma cells (0.02 ± 0.001 pmol/nmol phospholipid), and

human breast cancer MCF7 cells (0.05 ± 0.001 pmol/nmol phospholipid), which might explain their high resistance to apoptotic stimuli.

De novo synthesis of ceramide has been implicated in apoptosis of LNCaP cells induced by treatment with phorbol ester (15). Thus it was of interest to examine whether the increased ceramide levels was due to stimulation of ceramide synthase. mycotoxin fumonisin B1, a known inhibitor of ceramide synthase FB1 itself did not induce apoptosis, nor did it affect the extent of apoptosis induced by TNF and γ-irradiation (Fig. 5B), although in agreement with a recent study (15), it almost completely blocked the increase in TPA-induced apoptosis in LNCaP cells. These results suggest that the de novo ceramide generation pathway does not play a role in apoptosis induced by TNF-α and γ-irradiation. Ceramide increase preceded that of sphingosine, suggesting that the increase in sphingosine might result from degradation of the ceramide. This is a likely possibility because sphingosine is not synthesized *de novo*, and can only be produced by metabolism of ceramide (39),. However, the ceramidase inhibitors (N-oleoylethanolamine (NOE), a proposed acidic ceramidase inhibitor, by itself even at relative low concentration (0.1 mM) markedly induced cell death. Although, it should be pointed out that NOE may not be a specific acidic ceramidase because it did not inhibit acidic ceramidase activity in an in vitro assay.

Sphingosine and N,N-dimethylsphingosine sensitize LNCaP cells to γ-irradiation-induced apoptosis

Sphingosine, but not ceramide, induced apoptosis of the androgen-independent human prostatic carcinoma cell line DU-145 (17, 20). To further examine whether sphingosine generation might also be important to sensitize LNCaP cells to irradiation, we used exogenously added sphingosine which is efficiently taken up by cells. Significant apoptosis was induced by treatment with 20 μM sphingosine which was detectable only after 72 h (Fig. 6). Sphingosine also markedly sensitized LNCaP cells to γ-radiation in a dose-dependent manner (Fig. 6) which was evident even at 48 h. After irradiation in the presence of 20 μM sphingosine, most of the cells were apoptotic (>60%) by 72 h.

Another means to increase sphingosine levels is by inhibition of sphingosine kinase. Recently, we (30) and others (31) have shown that N,N-dimethylsphingosine (DMS) is a specific competitive inhibitor of sphingosine kinase which is effective at concentrations that do not inhibit protein kinase C. DMS, at concentrations of 10 and 20 μM, induced 20% and 60% apoptosis, respectively, after72 h (Fig. 7). Moreover, DMS also sensitized LNCaP to apoptosis induced by γ-irradiation in a dose-dependent manner.

Activation of caspases in sphingosine and N,N-dimethylsphingosine-induced sensitization of LNCaP cells to γ-irradiation

The broad specificity tetrapeptide caspase inhibitor Z-VAD-FMK blocks apoptosis induced by sphingosine or DMS in HL-60 (32) and Hep3B hepatoma cells (19), suggesting that a protease of the caspase-3 subfamily is activated. Thus, we examined whether elevation of sphingosine resulted in activation of caspases-3 and -7, which drive the effector phase of apoptosis by cleaving key proteins, particularly the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). As previously shown (16), treatment with 30 nM okadaic acid for 48 h resulted in apoptosis and PARP cleavage in LNCaP cells (Fig. 8). In agreement with our previous studies (16), when compared to okadaic acid, sphingolipid metabolites were less effective in inducing PARP cleavage, even in the presence of radiation. Barely detectable PARP cleavage was seen after cells were exposed to sphingosine alone at 20 μM, a concentration that induced 30% apoptosis at 72 h (Fig. 6). PARP cleavage activity was increased in cells treated with sphingosine and 8 Gy irradiation after 72 h (Fig. 8), in agreement with the enhanced apoptosis. Interestingly, DMS alone was able to induce PARP cleavage in LNCaP without irradiation (Fig. 8), in agreement with its ability to induce apoptosis. Proteolytic processing of procaspases-3 and -7 was examined by Western-blotting using anti-sera specific for caspase-3 and the active p20 caspase-7 subunit, respectively (Fig. 8). In agreement with our previous study (16), the procaspases-3 and -7 were cleaved into their active forms after treatment with okadaic acid, whereas no significant activation of caspase-3 could be detected in extracts from irradiated cells treated with sphingosine or DMS (Fig. 8). However, activation of caspase-7 in the presence of sphingosine and DMS, especially after irradiation, was detected by appearance of the 20 kD large subunit (Fig. 8). In contrast, no activation of the initiator caspase-8 could be detected after treatment with sphingosine or DMS, in the absence or presence of irradiation (Fig. 8).

DISCUSSION

Recently, we and others have suggested that the sphingolipid metabolites, ceramide and sphingosine, provide proapoptotic signals, and a further metabolite, SPP, promotes cell survival and suppresses apoptosis (6, 7, 33). Dysregulation of this sphingolipid biostat may be important in the acquisition of malignant phenotypes and radioresistance in which transformed cells can circumvent existing apoptotic mechanisms that would normally target the destruction of these cells. Thus, resetting this biostat could potentially be used to enhance apoptosis and overcome resistance to radiation or androgen ablation.

Previously, it has been suggested that LNCaP cells are highly resistant to induction of apoptosis by γ–irradiation due in part to a defect in ceramide generation (15, 16). Likewise,

resistance to apoptosis involves a defect in ceramide generation in the PC3 prostate cancer cell line . Although in LNCaP cells, irradiation did not result in ceramide generation or apoptosis, pretreatment with TPA not only enhanced radiation-induced apoptosis, but also enabled ceramide generation via activation of ceramide synthase (34). In agreement, apoptosis was abrogated by fumonisin B1, a competitive inhibitor of ceramide synthase. Most importantly, when transplanted orthotopically into the prostate of nude mice, LNCaP cells produced tumors that showed the same responses to TPA and radiation therapy (34). However, apoptosis induced by treatment with TNF and γ-irradiation was not mediated by stimulation of de novo ceramide synthesis. Similarly, apoptosis in LNCaP cells induced by the topoisomerase 1 inhibitor camptothecin, ceramide generation was also independent of the de novo pathway.

A further metabolite of ceramide, sphingosine, has also been shown to induce apoptosis of androgen-independent human prostate cancer cells (20). Indeed, we found that ceramide production after TNF-α treatment in irradiated LNCaP cells or after SMase treatment is followed by a surge in sphingosine preceding caspase activation and the onset of apoptosis. Furthermore, while addition of exogenous sphingosine induced modest apoptosis by itself, it significantly sensitized LNCaP cells to γ-irradiation. Irradiation of TSU-Pr1 cells, but not LNCaP cells, also produced a marked decrease in the activity of sphingosine kinase, the enzyme that phosphorylates sphingosine to form SPP, with a corresponding increase in sphingosine levels. In addition, a correlation between cell death and decreased SPP levels was observed in LNCaP cells treated with TNF-α plus γ-irradiation. Interestingly, SPP levels also decreased after treatment with TNF-α alone which induces only modest elevations of sphingosine or ceramide, suggesting that the balance between these sphingolipid metabolites regulates LNCaP cell survival. These results raise the possibility that the individual enzymes in sphingolipid metabolism can be differentially regulated. It has recently been shown that sphingosine kinase can be activated independently of sphingomyelinase or ceramidase (35). Furthermore, inhibition of sphingosine kinase by DMS blocked the increase in SPP, induced apoptosis, and sensitized prostate cancer cells to γ-irradiation. Thus, the regulation of the sphingolipid biostat may also have important implications for the treatment of prostate cancer, since many therapeutic approaches have been shown to cause accumulation of ceramide and sphingosine, including chemotherapy and ionizing radiation (6, 7, 33).

Ceramide generation in response to apoptotic stimuli is complex and it could arise by degradation of sphingomyelin as a result of activation of neutral or acidic SMase or via de novo synthesis by stimulation of ceramide synthase (6, 7). In LNCaP cells, neither SMase nor ceramide synthase are activated after irradiation alone (36). In agreement, we failed to detect changes in ceramide levels in γ-

irradiated LNCaP (Fig. 2 and (16)). However, TNF-α induced a ~2-fold ceramide elevation that was potentiated by irradiation. TNF-α recruitment of the adaptor protein FADD is required for stimulation of acidic SMase (37), suggesting a possible mechanism for ceramide generation in LNCaP cells. Alternatively, ceramide can be generated by activation of ceramide synthase as has been shown for apoptosis in LNCaP cells mediated by PKC activation induced by the phorbol ester TPA (15). Interestingly, sphingosine which has been shown to inhibit PKC in several types of cells (38, 39), triggers apoptosis in LNCaP cells, thus excluding the possibility that sphingosine is acting through PKC inhibition in LNCaP cells. The effects of TNF-α, like those of TPA, are pleiotropic (40). TNF-α has a pro-apoptotic effect in numerous tumor cells or virally- infected cells; whereas in some normal cells, such as in human endothelial cells, TNF-α is not cytotoxic. A recent study demonstrated that in these cells, TNF-α simultaneously and independently regulated sphingomyelinase and the sphingosine kinase activity, and suggested that the balance of these two antagonistic biochemical signaling pathways could regulate the fate of cell in response to TNF-α stimulation (35).

Protease inhibitor studies indicate that ceramide and sphingosine may act independently to induce cell death, and suggest that sphingosine induces activation of upstream caspases (19, 32). However, ceramide generated by upstream caspases, has been shown to activate additional downstream caspases necessary for apoptosis (6, 41-45). Moreover, ceramide could also induce cell death by a caspase-independent pathway (46). We found that caspase-7, but not caspase-3, was activated in sphingosine-sensitized γ-irradiation-induced apoptosis of LNCaP cells, in agreement with previous reports suggesting that activation of caspase-7, but not caspase-3, is an important step in the execution of apoptosis in LNCaP cells (47). The fact that LNCaP cell lines stably overexpressing crmA are resistant to sphingolipid induced-apoptosis (Kimura et al, unpublished data) may place crmA inhibitable-caspases downstream of sphingolipid metabolites. Interestingly, elevation of ceramide and/or sphingosine in LNCaP cells only had a modest effect on caspase activation ((16) and Fig. 7), whereas okadaic acid induced robust activation of several caspases while triggering a similar extent of apoptosis in comparison with γ-irradiation combined with TNF-α or with sphingolipid metabolites. This underscores the specificity of the apoptotic response to different stimuli in LNCaP cells, and may indicate the participation of other death proteases, such as serine proteases, that cooperate with caspases in the execution of cell death (16, 48). This might be especially important for sensitization of radiation-induced apoptosis by sphingosine and DMS treatments, because they were able to enhance apoptosis with a limited degree of caspase activation.

Different caspases are known to be activated in LNCaP and TSU-Pr1 cells, depending on the apoptotic stimuli. In LNCaP cells, caspases-3,-6,-7, and -8 are activated after okadaic acid treatment. In contrast, caspase-3 is not involved in TNF-α or γ-radiation-induced death in LNCaP cells, but is markedly activated in TSU-Pr1 cells. This could explain the fact that LNCaP cells are highly resistant to radiation-induced apoptosis compared to TSU-Pr1 cells (16, 49). Recent studies have demonstrated that caspase-7 and -3 are critical mediators of apoptosis in LNCaP cells. Moreover, overexpression of caspase-7 induced apoptosis even in LNCaP cells overexpressing the oncoprotein bcl-2 (49). However, in addition to the differential activation of unique caspases (16), our recent study suggests the involvement of additional cell-type specific signaling events in prostate cancer cell death, including activation of the JNK/SAPK pathway (27), which is thought to be involved in ceramide (50) and sphingosine-induced apoptosis (51). Interestingly, expression of bcl-2 not only protected prostate carcinoma cells against the induction of apoptosis by exogenous C2-ceramide but also blocked its ability to activate JNK1, indicating bcl-2 functions at the level of JNK1 or upstream of JNK1 in the ceramide/JNK pathway (52).

Collectively, our results suggest that ceramide and sphingosine generation together with inhibition of sphingosine kinase are critical components in radiation-induced apoptosis in human prostate cancer cells. Preventing ceramide and sphingosine generation and/or stimulation of sphingosine kinase may provide a selective advantage in the development of radioresistance of prostate tumors. Therefore, development of agents which specifically regulate levels of sphingolipid metabolites might provide new tools to use in conjunction with radiation therapy.

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FIGURES

See proofs of the paper in press in Cancer Research.

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APPENDICES

[CANCER RESEARCH 60, 0000–0000, August 15, 2000]

Sphingosine Enhances Apoptosis of Radiation-resistant Prostate Cancer Cells[1]

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ABSTRACT

Ceramide has been implicated as an important component of radiation-induced apoptosis of human prostate cancer cells. We examined the role of the sphingolipid metabolites—ceramide, sphingosine, and sphingosine-phosphate—in susceptibility to radiation-induced apoptosis in prostate cancer cell lines with different sensitivities to γ-irradiation. Exposure of radiation-sensitive TSU-Pr1 cells to 8-Gy irradiation led to a sustained increase in ceramide, beginning after 12 h of treatment and increasing to 2.5- to 3-fold within 48 h. Moreover, irradiation of TSU-Pr1 cells also produced a marked and rapid 50% decrease in the activity of sphingosine kinase, the enzyme that phosphorylates sphingosine to form sphingosine-1-phosphate. In contrast, the radiation-insensitive cell line, LNCaP, had sustained sphingosine kinase activity and did not produce elevated ceramide levels on 8-Gy irradiation. Although LNCaP cells are highly resistant to γ-irradiation-induced apoptosis, they are sensitive to the death-inducing effects of tumor necrosis factor α which also increases ceramide levels in these cells (K. Kimura *et al.*, Cancer Res., *59:* 1606–1614, 1999). Moreover, we found that although irradiation alone did not increase sphingosine levels in LNCaP cells, tumor necrosis factor α plus irradiation induced significantly higher sphingosine levels and markedly reduced intracellular levels of sphingosine-1-phosphate. The elevation of sphingosine levels either by exogenous sphingosine or by treatment with the sphingosine kinase inhibitor *N,N*-dimethylsphingosine induced apoptosis and also sensitized LNCaP cells to γ-irradiation-induced apoptosis. Our data suggest that the relative levels of sphingolipid metabolites may play a role in determining the radiosensitivity of prostate cancer cells, and that the enhancement of ceramide and sphingosine generation could be of therapeutic value.

INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer deaths in men (1). Radiation therapy that causes growth inhibition and apoptosis is often used for treatment of both primary and metastatic prostate cancer. However, despite using high doses of radiation, about 20–25% of prostate cancer patients with noninvasive disease (stages T_1-T_2) relapse. A major reason for failure to eradicate local disease is the intrinsic radioresistance of the tumors. Ionizing radiation mediates cell death, in part, through chromosomal damage and also by the induction of apoptosis. Although apoptosis seems to be less prevalent than clonogenic cell death, one mechanism by which cancer cells become resistant to radiation or chemotherapy is by the disruption of pathways leading to apoptosis.

Abundant evidence suggests that the sphingolipid metabolite, ceramide, is a critical component of ionizing radiation-induced apoptosis (2–5). This apoptotic pathway is initiated by hydrolysis of sphingomyelin, a membrane lipid, attributable to the activation of sphingomyelin-specific forms of phospholipase C, termed sphingomyelinases SMases,[3] to generate ceramide. Ceramide, in turn, can activate several pathways important for the induction of apoptosis (reviewed in Refs. 6, 7). Both neutral and acidic SMases, distinguishable by their pH optima, have been reported to be involved in the induction of apoptosis after ionizing radiation (reviewed in Refs. 8, 9). Acidic SMase may play an essential role in radiation-induced apoptosis because lymphocytes from individuals with Niemann-Pick disease (who have an inherited deficiency of acidic SMase) and from acidic SMase-deficient mice, do not generate ceramide or else have defective apoptotic responses to ionizing radiation (4). These deficits are reversible on restoration of acidic SMase activity, which further substantiates the obligatory role for ceramide generation in these apoptotic responses. However, ionizing radiation-triggered apoptosis of sensitive, but not resistant, human myeloid leukemic cell lines correlated with sphingomyelin hydrolysis and ceramide generation through activation of neutral, but not acidic, SMase (10). Similarly, loss of ceramide production from a neutral SMase confers resistance to radiation-induced apoptosis of lymphocytes (5). Moreover, depletion of glutathione, an endogenous inhibitor of neutral SMase (11), may also contribute to its activation, because glutathione depletion occurs in a variety of cells during radiation-induced apoptosis (12, 13). In addition, it has been suggested that *de novo* synthesis of ceramide as a result of increased ceramide synthase activity may also be involved in apoptosis (14), particularly in radiation-insensitive LNCaP prostate cancer cells that are induced to die by the phorbol ester PMA (15). LNCaP cells express androgen receptor, and their growth is increased by androgen. However, because they do not undergo apoptosis after androgen withdrawal, these cells can be used as an *in vitro* model to study strategies for treating prostate cancers that are resistant to androgen ablation. LNCaP cells are highly resistant to apoptosis induced by γ-irradiation, although somewhat sensitive to the death-inducing effects of TNF-α. Recently, we have shown that TNF-α sensitizes LNCaP cells to γ-irradiation-induced apoptosis by elevating ceramide levels (16). Moreover, exogenous C_2-cer also sensitized LNCaP cells to irradiation, which lends further support to the notion that ceramide generation might be important for radiation-induced apoptosis in human prostate cancer.

One metabolite of ceramide, sphingosine, formed by ceramidase, has also been implicated in cell growth arrest and apoptosis. Sphingosine is rapidly produced during TNF-α-mediated apoptosis in human neutrophils (17) and cardiac myocytes (18). Recently, it has been shown that sphingosine and other long-chain sphingoid bases induce apoptosis in hepatoma cells by the activation of caspase-3-like proteases (19). Moreover, in androgen-independent human prostatic carcinoma DU-145 cells that express bcl-X_L, sphingosine but not its metabolites induced apoptosis by down-regulation of bcl-X_L, independently of PKC inhibition (20). In contrast to the growth-suppressing and pro-apoptotic roles of ceramide

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[3] The abbreviations used are: SMase, sphingomyelinase; TNF-α, tumor necrosis factor α; C_2-cer, C_2-ceramide (*N*-acetylsphingosine); IMEM, Richter's improved minimal essential medium; ISEL, *in situ* end labeling; PKC, protein kinase C; SPP, sphingosine-1-phosphate; DMS, *N,N*-dimethylsphingosine; PMA, phorbol 12-myristate 13-acetate;

and sphingosine, SPP, formed from sphingosine by activation of sphingosine kinase (21), has been implicated in cellular proliferation and survival induced by PDGF, serum, NGF, and vitamin D3, and protects cells from apoptosis resulting from elevations of ceramide (22–26). In this report, we examined the role of ceramide, sphingosine, and sphingosine kinase in the sensitization of radio-resistant LNCaP prostate cells to γ-irradiation induced apoptosis.

MATERIALS AND METHODS

Materials. Human TNF-α and poly-D-lysine were purchased from Boehringer Mannheim (Indianapolis, IN). *Staphylococcus aureus* SMase and FB1 were purchased from Sigma (St, Louis, MO). Sphingosine and DMS were from Biomol Laboratories (Plymouth Meeting, PA). *Escherichia coli* diacylglycerol kinase was from Calbiochem (La Jolla, CA). Insulin/tranferrin/selenium was from Biofluids (Rockville, MD).

Cell Culture. LNCaP and TSU-Pr1 human prostate cancer cells were maintained at 37°C in IMEM (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (27). LNCaP cells were plated on poly-D-lysine-coated dishes and grown for 5 days. Twenty-four h before treatment, cells were starved in serum-free IMEM medium without phenol red, supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml). Cells were treated as indicated without or with γ-irradiation (8 Gy), using a JL Shepherd Mark I Irradiator [^{137}Cs] source with a dose rate of 209 cGy/min.

Apoptosis Measurement. ISEL was used to determine the extent of apoptosis as described previously (27). In some experiments, apoptotic morphology was also examined by staining cells with Hoechst 33258 (Calbiochem, San Diego, CA) as previously described (24). At least 500 cells were scored to calculate the percentage of apoptotic cells.

Extraction of Lipids. Cells were harvested in 1 ml of 25 mM HCl/methanol, and lipids were extracted with 2 ml of chloroform/1 M NaCl (1:1, v/v) plus 100 μl 3N NaOH for the extraction of SPP and phases separated. Phospholipid, ceramide, and sphingosine levels were determined in aliquots of the organic layer, whereas SPP levels were determined from aqueous phase extracts (23).

Measurement of Total Cellular Phospholipids. Total phospholipids in cellular lipid extracts were quantified as described previously (28).

Measurements of Ceramide, Sphingosine, and SPP Levels. Mass amounts of ceramide and sphingosine in cellular extracts were measured by the diacylglycerol kinase and sphingosine kinase enzymatic methods, respectively, exactly as described previously (23). Labeled ceramide-1-phosphate and SPP were resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1) and quantified with a Molecular Dynamics Storm phosphoimager (Sunnyvale, CA). SPP levels were measured essentially as described previously (29). Briefly, 500 μl of buffer A [200 mM Tris-HCl (pH 7.4), 75 mM $MgCl_2$ in 2 M glycine (pH 9.0)] and 50 units of alkaline phosphatase were added to the aqueous phase containing extracted SPP. After incubating 1 h at 37°C, 50 μl concentrated HCl were added, and sphingosine was extracted and quantitated with sphingosine kinase as described previously (29). For each experiment, known amounts of SPP were used to generate a standard curve.

Sphingosine Kinase Activity. Cells were harvested in buffer B [20 mM Tris (pH 7.4), 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM -glycerophosphate, 0.5 mM 4-deoxypyridoxine, 15 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride] and lysed by freeze-thawing. Supernatants were collected after centrifugation at 100,000 × *g* for 30 min at 4°C. Cytosolic sphingosine kinase activity was determined as described previously (23).

Immunoblotting. Cells were harvested in buffer C [10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 20 μg/ml leupeptin] and Western blotting was carried out as described previously (24). Clone 7D3–6 mouse monoclonal anti-PARP (PharMingen, San Diego, CA; 0.5 μg/ml), rabbit polyclonal anti-caspase-7 (Oncogene, Cambridge, MA; 2.5 μg/ml), rabbit anti-caspase-3 (gift of Dr. Donald Nicholson), and mouse anti-caspase-8 (gift of Dr. Markus Peter), were used as primary antibodies. Proteins were visualized with SuperSignal-enhanced chemiluminescent rea-

RESULTS

Sphingomyelinase Treatment Sensitizes LNCaP Cells to γ-Irradiation-induced Apoptosis. Previously, we have shown that treatment with C_2-cer synergizes with γ-irradiation to induce cell death in LNCaP cells (16). In agreement, we have found that increasing endogenous long-chain ceramide levels in LNCaP cells by pretreatment with SMase induces apoptosis and sensitizes the cells to a dose of γ-irradiation (8 Gy) sufficient to trigger apoptosis of TSU-Pr1 but not of LNCaP cells (Fig. 1). Apoptosis increased in a time-dependent manner, and at least 48 h were required for significant cell death. To confirm the induction of apoptosis, we also examined DNA fragmentation and nuclear condensation by staining with the DNA-specific fluorochrome bisbenzimide (Hoechst 33258). Substantially more DNA ladder formation and fragmented nuclei were seen after 72 h than after 48 h (data not shown).

To examine whether there was a correlation between ceramide levels and radiation sensitivity, we measured changes in ceramide levels after irradiation of these two cell lines. Exposure of radiation-sensitive TSU-Pr1 cells to 8 Gy irradiation led to acute, but small, increases in ceramide levels within 30 min, followed by a sustained elevation in ceramide 12 h after irradiation, which reached a 3-fold increase by 48 h (Fig. 2*A*). This generation of ceramide preceded the appearance of nuclear fragmentation that was evident only after 48 h (Fig. 1). In contrast, ceramide levels did not change in radio-resistant LNCaP cells (Fig. 2*A*). In addition, irradiation induced a rapid and sustained decrease in sphingosine kinase activity in TSU-Pr1cells but not in LNCaP cells (Fig. 2*B*). A surge in sphingosine levels was detected in TSU-Pr1 cells in correspondence with the inhibition of sphingosine kinase (Fig. 2*C*). SPP levels in the radiosensitive TSU-Pr1 cells were below the detection limit (<0.01 pmol/nmol phospholipid), and we were, thus, unable to detect any increases after irradiation. Interestingly, levels of SPP in TSU-Pr1 cells were much lower than in LNCaP cells (0.12 pmol/nmol phospholipid), which are more resistant to γ-radiation. Thus, there seems to be a reciprocal relationship between ceramide/sphingosine and sphingosine kinase in radiation-sensitive prostate cancer cells.

Sphingosine Generation in Apoptosis-sensitized LNCaP Cells. Because it has been suggested that sphingosine, a breakdown product of ceramide, might also be a mediator of programmed cell death (19), it was of interest to determine whether sphingosine could be involved in the sensitization of LNCaP cells to apoptosis that was induced by irradiation. Treatment with bacterial SMase, which markedly sensitizes these cells to radiation (Fig. 1), as expected, induced a rapid 4- to 5-fold elevation in ceramide levels, which remained elevated at 24 h and declined thereafter (Fig. 3*A*). Interestingly, SMase treatment

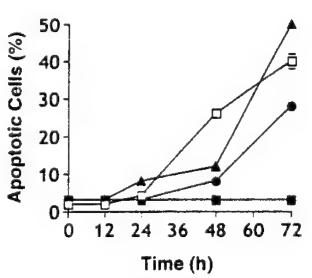

Fig. 1. Effect of γ-irradiation and sphingomyelinase on apoptosis in prostate cancer cells. LNCaP (*filled symbols*) and TSU-Pr1 (□) cells (1.5 × 10^4 per well) were seeded in 6-well plates coated with poly-D-lysine and grown until ~40% confluent. Cells were treated without (□, ■) or with 100 mU/ml SMase (▲, ●) for 1 h and then irradiated with 8 Gy (□, ■, ▲) as indicated. Apoptotic cells were assayed by ISEL at the indicated times, as described in "Materials and Methods." Results are means ± SD of triplicate determi-

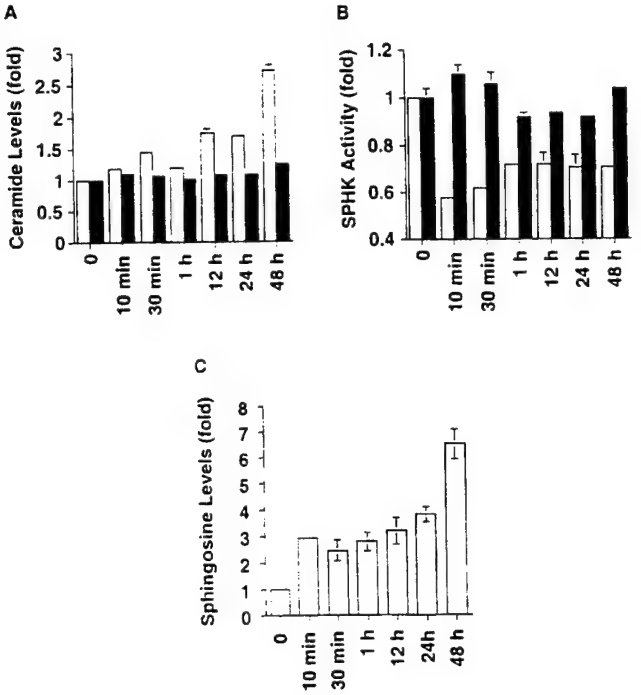

Fig. 2. γ-Irradiation-induced ceramide elevation and decreased sphingosine kinase activity in TSU-Pr1 but not in LNCaP cells. LNCaP cells (■) or TSU-Pr1 cells (□) were seeded at 2×10^5 per well and grown in 10-cm dishes. At the indicated time after exposure to 8 Gy of irradiation, ceramide levels (*A*), sphingosine kinase activity (*B*), and sphingosine levels (*C*, □), were measured as described in "Materials and Methods." Results are means ± SD of triplicate determinations from a representative experiment and are expressed as fold-changes relative to zero time. The basal sphingosine level in TSU-Pr1 cells was 0.23 ± 0.07 pmol/nmol phospholipid. Similar results were found in three independent experiments. However, the small acute ceramide increase at 30 min observed in *A* was not statistically significant. □, TSU-Pr1 (8 Gy); ■, LNCap (8 Gy).

γ-irradiation (Fig. 5*B*), although in agreement with a recent study (15), it almost completely blocked the increase in PMA-induced apoptosis in LNCaP cells. These results suggest that the *de novo* ceramide generation pathway does not play a role in the apoptosis that is induced by TNF-α and γ-irradiation. The increase in ceramide

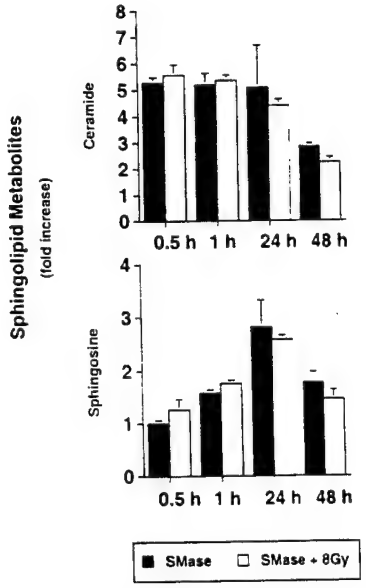

Fig. 3. Changes in ceramide and sphingosine levels after γ-irradiation of sphingomyelinase-treated LNCaP cells. Cells were seeded and grown as described in Fig. 2 and treated without or with SMase (100 mU/ml) and then irradiated with 8 Gy as indicated. At the indicated times, ceramide, sphingosine, and phospholipid levels were determined as described in "Materials and Methods." Data are expressed as fold-increases relative to untreated controls, and are means ± SD of triplicate determinations from a representative experiment.

also increased intracellular sphingosine after 24 h (Fig. 3*B*). It is likely that this increase in sphingosine results from degradation of ceramide, inasmuch as the ceramide increase preceded that of sphingosine. It should be pointed out that the elevation of these two sphingolipid metabolites precedes the onset of apoptosis (Fig. 1). As previously shown (16), we found that TNF-α also sensitized LNCaP cells to irradiation with a concomitant increase in ceramide levels (Fig. 4*A* and 5*A*). Although irradiation alone did not increase sphingosine levels in LNCaP cells, irradiation together with TNF-α induced significantly higher sphingosine levels than TNF-α alone (Fig. 4*B*). The increase in sphingosine was detected 24 h after treatment, coinciding with elevation in ceramide levels and preceding nuclear fragmentation that was evident 48 h after treatment (16). In LNCaP cells that were sensitized to die by irradiation and TNF-α, there was a marked decrease in SPP levels 48 h after treatment, whereas no significant changes in SPP levels were detected in cells treated with γ-irradiation alone (Fig. 4*C*). It should be noted that SPP levels in LNCaP cells (0.12 ± 0.01 pmol/nmol phospholipid) are greater than in other normal and cancer cells (29), including HL60 cells (0.01 ± 0.001 pmol/nmol phospholipid), PC12 pheochromocytoma cells (0.02 ± 0.001 pmol/nmol phospholipid), and human breast cancer MCF7 cells (0.05 ± 0.001 pmol/nmol phospholipid), which might explain the high resistance of LNCaP cells to apoptotic stimuli.

De novo synthesis of ceramide has been implicated in the apoptosis of LNCaP cells induced by treatment with phorbol ester (15). Thus, it was of interest to examine whether the increased ceramide levels was attributable to stimulation of ceramide synthase. The mycotoxin, FB1, a known inhibitor of ceramide synthase, alone did not induce apoptosis, nor did it affect the extent of apoptosis induced by TNF and

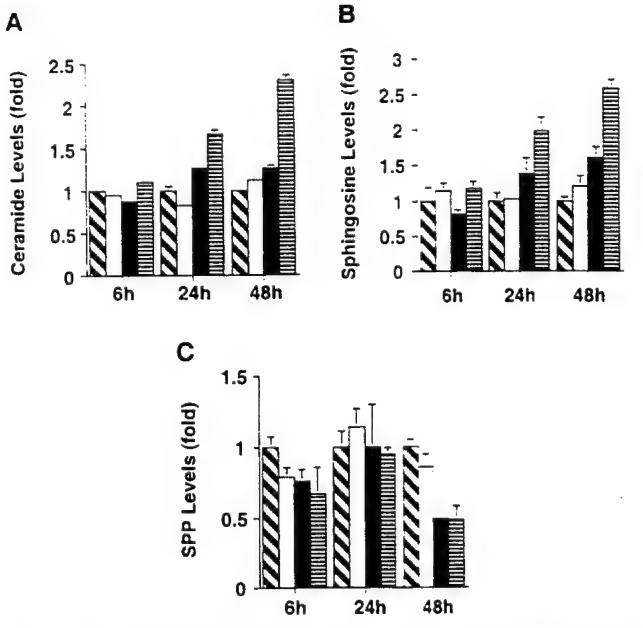

Fig. 4. Changes in levels of the sphingolipid metabolites—ceramide, sphingosine, and SPP—after treatment of LNCaP cells with γ-irradiation and TNF-α. Cells were seeded and grown as described in Fig. 2. Cells were treated without (control) or with TNF-α (100 ng/ml) for 1 h before irradiation with 8 Gy as indicated. Ceramide (*A*), sphingosine (*B*), SPP (*C*) and phospholipid levels were measured after 6, 24, and 48 h as described in "Materials and Methods." Results are means ± SD of triplicate determinations from a representative experiment and are expressed as fold-changes relative to control. Levels of ceramide, sphingosine and SPP in untreated LNCaP cells were 19 ± 2, 0.23 ± 0.03, and 0.29 ± 0.06 pmol/nmol phospholipid, respectively. ▨, control; □ γ radiation (8 GY); ■, TNF-α; ▤, TNF-α + 8 Gy.

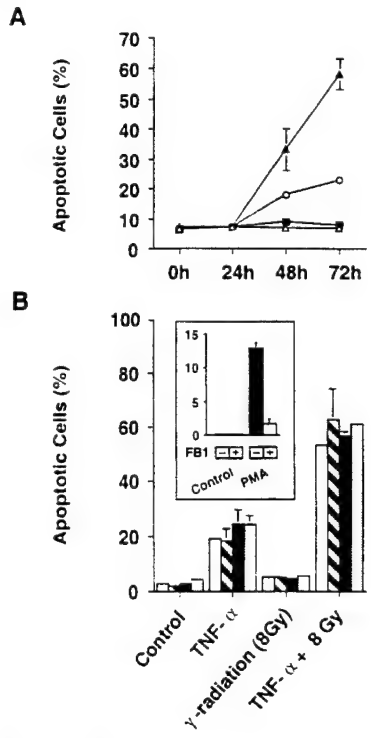

Fig. 5. Effect of FB1 on TNF-α and γ-irradiation-induced apoptosis. In *A*, cells were treated without (*squares*) or with 100 ng/ml TNF-α (▲, ○) followed by irradiation (▲, ■), and apoptosis was assayed by ISEL at the indicated times. ▲, TNF-α + 8 Gy; ○, TNF-α; ■, γ radiation (8 Gy); △, control. *B*, cells were treated with the indicated concentrations of FB1 in the absence or presence of TNF-α and/or 8 Gy irradiation as indicated, and apoptosis was determined after 72 h. □, 0 μM FB1; ▨, 50 μM FB1; ■, 100 μM FB1; □, 150 μM FB1. *Inset*, the effect of FB1 (100 μg/ml) on PMA-induced apoptosis 48 h after treatment.

preceded that of sphingosine, which suggested that sphingosine might arise from ceramidase-catalyzed metabolism of ceramide. This is a likely possibility because sphingosine is not synthesized *de novo*, and can only be produced from ceramide (30, 31). However, *N*-oleoylethanolamine, a proposed acidic ceramidase inhibitor (32), by itself, even at a relatively low concentration (0.1 mM), markedly induced LNCaP cell death. It should be pointed out that *N*-oleoylethanolamine may not be a specific acidic ceramidase because it did not inhibit acidic ceramidase activity in an *in vitro* assay.

Sphingosine and DMS Sensitize LNCaP Cells to γ-Irradiation-induced Apoptosis. Sphingosine, but not ceramide, induced apoptosis of the androgen-independent human prostatic carcinoma cell line DU-145 (17, 20). To further examine whether sphingosine generation might also be important to sensitize LNCaP cells to irradiation, we used exogenously added sphingosine, which is efficiently taken up by cells. Significant apoptosis was induced by treatment with 20 μM sphingosine that was detectable only after 72 h (Fig. 6). Sphingosine also markedly sensitized LNCaP cells to γ-radiation in a dose-dependent manner (Fig. 6), which was evident even at 48 h. After irradiation in the presence of 20 μM sphingosine, most (>60%) of the cells were apoptotic by 72 h.

Another means to increase sphingosine levels is by the inhibition of sphingosine kinase. Recently, we (33) and others (34) have shown that DMS is a specific competitive inhibitor of sphingosine kinase which is effective at concentrations that do not inhibit PKC. DMS, at concentrations of 10 and 20 μM, induced 20 and 60% apoptosis, respectively, after 72 h (Fig. 7). Moreover, DMS also sensitized LNCaP cells to apoptosis induced by γ-irradiation in a dose-dependent manner

Activation of Caspases in Sphingosine and DMS-induced Sensitization of LNCaP Cells to γ-Irradiation. The broad-specificity tetrapeptide caspase inhibitor Z-VAD-FMK blocks apoptosis induced by sphingosine or DMS in HL-60 (35) and Hep3B hepatoma cells (19), which suggests that a protease of the caspase-3 subfamily is

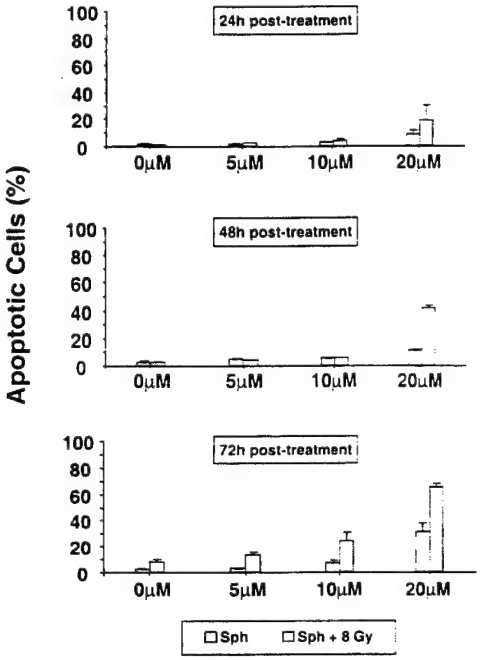

Fig. 6. Sphingosine sensitizes LNCaP cells to γ-irradiation-induced apoptosis. LNCaP cells were treated without or with the indicated concentrations of sphingosine for 1 h and then either not irradiated (□) or irradiated with 8 Gy (□). Apoptotic cells were assayed by ISEL at the indicated times. Data are means ± SD of triplicate determinations from one of three representative experiments.

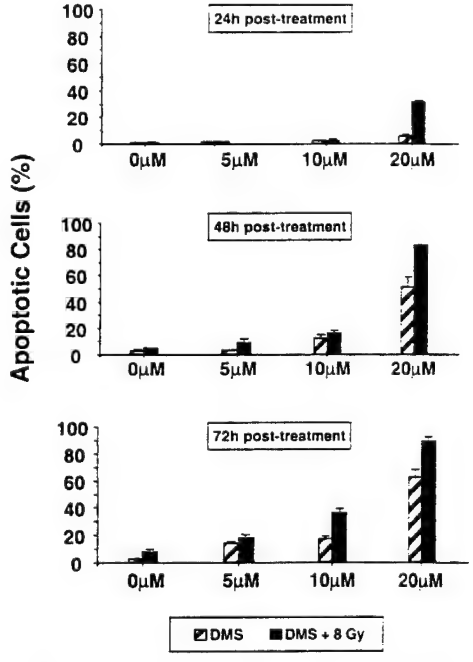

Fig. 7. The sphingosine kinase inhibitor, DMS, induces apoptosis and sensitizes LNCaP cells to γ-irradiation-induced apoptosis. LNCaP cells were treated without or with the indicated concentrations of DMS for 1 h and then either not irradiated (▲) or irradiated with 8 Gy (■). Apoptotic cells were assayed by ISEL at the indicated times. Data are means ± SD of triplicate determinations from one of three representative experiments.

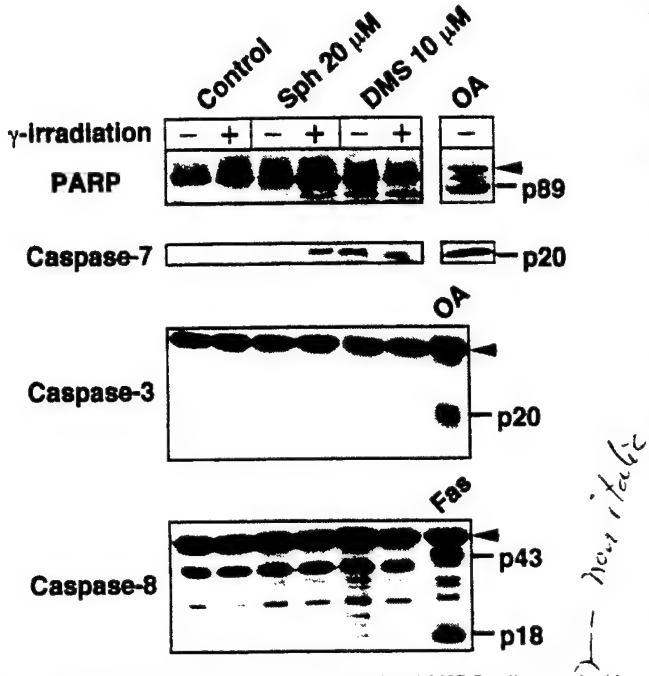

Fig. 8. Cleavage of PARP and caspase-7 in γ-irradiated LNCaP cells treated with sphingosine or DMS. LNCaP cells were treated for 72 h with 20 μM sphingosine (*Sph*) or 10 μM DMS without or with 8 Gy γ-irradiation. Cell lysates were prepared and equal amounts of proteins were immunoblotted with anti-PARP, anti-caspase-7, anti-caspase-3, or anti-caspase-8 antibodies after separation by SDS-PAGE (10% SDS gels for PARP and 15% for the others). *Arrowheads*, the migration of full-length PARP, caspase-3, and caspase-8. *Left*, PARP cleavage product (M_r 89,000), the large active subunits of caspase-7 (M_r 20,000), caspase-3 (M_r 20,000), and caspase-8 (M_r 18,000), and the intermediate cleavage fragment of caspase-8 (M_r 43,000). Lysate from LNCaP cells treated with okadaic acid (30 nM) for 48 h served as positive control for cleavage of PARP, caspase-7 and caspase-3. H9 T cells treated with anti-Fas antibody (50 ng/ml) for 24 h were used as a positive control for caspase-8 processing. Similar results were obtained in three independent experiments.

activated. Thus, we examined whether elevation of sphingosine resulted in the activation of caspases-3 and -7, which drive the effector phase of apoptosis by cleaving key proteins, particularly the DNA repair enzyme PARP. As previously shown (16), treatment with 30 nM okadaic acid for 48 h resulted in apoptosis and PARP cleavage in LNCaP cells (Fig. 8). In agreement with our previous studies (16), when compared with okadaic acid, sphingolipid metabolites were less effective in inducing PARP cleavage, even in the presence of radiation. Barely detectable PARP cleavage was seen after cells were exposed to sphingosine alone at 20 μM, a concentration that induced 30% apoptosis at 72 h (Fig. 6). PARP cleavage activity was increased in cells treated with sphingosine and 8 Gy irradiation after 72 h (Fig. 8), in agreement with the enhanced apoptosis. Interestingly, DMS alone was able to induce PARP cleavage in LNCaP cells without irradiation (Fig. 8), in agreement with its ability to induce apoptosis. Proteolytic processing of procaspases-3 and -7 was examined by Western blotting using antisera specific for caspase-3 and the active p20 caspase-7 subunit, respectively (Fig. 8). In agreement with our previous study (16), the procaspases-3 and -7 were cleaved into their active forms after treatment with okadaic acid, whereas no significant activation of caspase-3 could be detected in extracts from irradiated cells treated with sphingosine or DMS (Fig. 8). However, the activation of caspase-7 in the presence of sphingosine and DMS, especially after irradiation, was detected by the appearance of the M_r 20,000 large subunit (Fig. 8). In contrast, no activation of the initiator caspase-8 could be detected after treatment with sphingosine or DMS.

DISCUSSION

Recently, we and others (6, 7, 36) have suggested that the sphingolipid metabolites, ceramide and sphingosine, provide proapoptotic signals, and an additional metabolite, SPP, promotes cell survival and suppresses apoptosis. Dysregulation of this sphingolipid biostat may be important in the acquisition of malignant phenotypes and radioresistance in which transformed cells can circumvent existing apoptotic mechanisms that would normally target the destruction of these cells. Thus, resetting this biostat could potentially be used to enhance apoptosis and overcome resistance to radiation or androgen ablation.

Previously, it has been suggested that LNCaP cells are highly resistant to induction of apoptosis by γ-irradiation attributable in part to a defect in ceramide generation (15, 16). Likewise, resistance to apoptosis involves a defect in ceramide generation in the PC3 prostate cancer cell line (37). However, radiation-induced apoptosis is not solely dependent on ceramide signaling, and there are other ceramide-independent pathways leading to apoptosis. Although in LNCaP cells, irradiation did not result in ceramide generation or apoptosis, pretreatment with PMA not only enhanced radiation-induced apoptosis, but also enabled ceramide generation via activation of ceramide synthase (38). In agreement, apoptosis was abrogated by FB1, a competitive inhibitor of ceramide synthase. Most importantly, when transplanted orthotopically into the prostate of nude mice, LNCaP cells produced tumors that showed the same responses to PMA and radiation therapy (38). However, apoptosis induced by treatment with TNF and γ-irradiation was not mediated by stimulation of *de novo* ceramide synthesis. Similarly, apoptosis in LNCaP cells induced by the topoisomerase 1 inhibitor camptothecin, ceramide generation was also independent of the *de novo* pathway (37).

A further metabolite of ceramide, sphingosine, has also been shown to induce apoptosis of androgen-independent human prostate cancer cells (20). Indeed, we found that ceramide production after TNF-α treatment in irradiated LNCaP cells or after SMase treatment is followed by a surge in sphingosine that precedes caspase activation and the onset of apoptosis. Furthermore, whereas addition of exogenous sphingosine induced modest apoptosis by itself, it significantly sensitized LNCaP cells to γ-irradiation. Irradiation of TSU-Pr1 cells, but not LNCaP cells, also produced a marked decrease in the activity of sphingosine kinase, the enzyme that phosphorylates sphingosine to form SPP, with a corresponding increase in sphingosine levels. In addition, a correlation between cell death and decreased SPP levels was observed in LNCaP cells treated with TNF-α plus γ-irradiation. Interestingly, SPP levels also decreased after treatment with TNF-α alone, which induces only modest elevations of sphingosine and ceramide, and which suggests that the balance between these sphingolipid metabolites may regulate LNCaP cell survival. These results raise the possibility that the individual enzymes in sphingolipid metabolism can be differentially regulated. In agreement, it has recently been shown that sphingosine kinase can be activated independently of sphingomyelinase or ceramidase (39). Furthermore, inhibition of sphingosine kinase by DMS blocked the increase in SPP, induced apoptosis, and sensitized prostate cancer cells to γ-irradiation. Thus, the regulation of the sphingolipid biostat may also have important implications for the treatment of prostate cancer, because many therapeutic approaches have been shown to cause accumulation of ceramide and sphingosine, including chemotherapy and ionizing radiation (6, 7, 36).

Ceramide generation in response to apoptotic stimuli is complex (6, 7). In LNCaP cells, neither SMase nor ceramide synthase are activated after irradiation alone (40). In agreement, we failed to detect changes in ceramide levels in γ-irradiated LNCaP cells (Fig. 2 and Ref. 16).

potentiated by irradiation. TNF-α recruitment of the adaptor protein FADD is required for stimulation of acidic SMase (41), which suggests a possible mechanism for ceramide generation in LNCaP cells. Alternatively, ceramide can be generated by the activation of ceramide synthase as has been shown to mediate apoptosis in LNCaP cells mediated by PKC activation induced by the phorbol ester PMA (15). Interestingly, sphingosine, which has been shown to inhibit PKC in several types of cells (30, 31), triggers apoptosis in LNCaP cells, thus excluding the possibility that sphingosine is acting through PKC inhibition in LNCaP cells. The effects of TNF-α, like those of PMA, are pleiotropic (42). TNF-α has a pro-apoptotic effect in numerous tumor cells or virally infected cells, whereas, in some normal cells, such as in human endothelial cells, TNF-α is not cytotoxic. A recent study demonstrated that in these cells, TNF-α simultaneously and independently regulated sphingomyelinase and sphingosine kinase activity, which leads to the suggestion that the balance of these two antagonistic biochemical signaling pathways could regulate the fate of cells in response to TNF-α stimulation (39).

Protease inhibitor studies indicate that ceramide and sphingosine may act independently to induce cell death, and it is argued that sphingosine induces activation of upstream caspases (19, 35). However, ceramide generated by upstream caspases, has been shown to activate additional downstream caspases necessary for apoptosis (6, 43–47). Moreover, ceramide could also induce cell death by a caspase-independent pathway (48). We found that caspase-7, but not caspase-3, was activated in sphingosine-sensitized γ-irradiation-induced apoptosis of LNCaP cells, in agreement with previous reports suggesting that activation of caspase-7, but not caspase-3, is an important step in the execution of apoptosis in LNCaP cells (49). The fact that LNCaP cell lines stably overexpressing crmA are resistant to sphingolipid induced-apoptosis[4] may place crmA-inhibitable caspases downstream of sphingolipid metabolites. Interestingly, elevation of ceramide and/or sphingosine in LNCaP cells only had a modest effect on caspase activation (Ref. 16) and Fig. 7), whereas okadaic acid induced robust activation of several caspases while triggering a similar extent of apoptosis in comparison with γ-irradiation combined with TNF-α or with sphingolipid metabolites. This underscores the specificity of the apoptotic response to different stimuli in LNCaP cells and may indicate the participation of other death proteases, such as serine proteases, that cooperate with caspases in the execution of cell death (16, 50). This may be especially important for sensitization of radiation-induced apoptosis by sphingosine and DMS, because they were able to enhance apoptosis with a limited degree of caspase activation.

Different caspases are known to be activated in LNCaP and TSU-Pr1 cells, depending on the apoptotic stimuli. In LNCaP cells, caspases-3, -6, -7, and -8 are activated after okadaic acid treatment. In contrast, caspase-3 is not involved in TNF-α- or γ-radiation-induced death in LNCaP cells, but is markedly activated in TSU-Pr1 cells. This could explain the fact that LNCaP cells are highly resistant to radiation-induced apoptosis compared with TSU-Pr1 cells (16, 51). Recent studies have demonstrated that caspase-7 and -3 are critical mediators of apoptosis in LNCaP cells. Moreover, overexpression of caspase-7 induced apoptosis even in LNCaP cells that overexpressed the oncoprotein bcl-2 (51). However, in addition to the differential activation of unique caspases (16), our recent study suggests the involvement of additional cell-type specific signaling events in prostate cancer cell death, including activation of the JNK/SAPK pathway (27), which is thought to be involved in ceramide (52) and sphingosine-induced apoptosis (53). Interestingly, expression of bcl-2 not only protected prostate carcinoma cells against the induction of apoptosis by exogenous C2-cer but also blocked its ability to activate JNK1, which indicated that bcl-2 functions at the level of JNK1 or upstream of JNK1 in the ceramide/JNK pathway (54).

Collectively, our results suggest that ceramide and sphingosine generation, together with the inhibition of sphingosine kinase, are critical components in radiation-induced apoptosis in human prostate cancer cells. Preventing ceramide and sphingosine generation and/or stimulation of sphingosine kinase may provide a selective advantage in the development of radioresistance of prostate tumors. Therefore, development of agents that specifically regulate levels of sphingolipid metabolites may provide new tools to use in conjunction with radiation therapy.

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Molecular Cloning and Functional Characterization of a Novel Mammalian Sphingosine Kinase Type 2 Isoform*

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Sphingosine-1-phosphate (SPP) has diverse biological functions acting inside cells as a second messenger to regulate proliferation and survival, and extracellularly, as a ligand for G protein-coupled receptors of the endothelial differentiation gene-1 subfamily. Based on sequence homology to murine and human sphingosine kinase-1 (SPHK1), which we recently cloned (Kohama, T., Oliver, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998) *J. Biol. Chem.* 273, 23722–23728), we have now cloned a second type of mouse and human sphingosine kinase (mSPHK2 and hSPHK2). mSPHK2 and hSPHK2 encode proteins of 617 and 618 amino acids, respectively, both much larger than SPHK1, and though diverging considerably, both contain the conserved domains found in all SPHK1s. Northern blot analysis revealed that SPHK2 mRNA expression had a strikingly different tissue distribution from that of SPHK1 and appeared later in embryonic development. Expression of SPHK2 in HEK 293 cells resulted in elevated SPP levels. D-*erythro*-dihydrosphingosine was a better substrate than D-*erythro*-sphingosine for SPHK2. Surprisingly, D, L-*threo*-dihydrosphingosine was also phosphorylated by SPHK2. In contrast to the inhibitory effects on SPHK1, high salt concentrations markedly stimulated SPHK2. Triton X-100 inhibited SPHK2 and stimulated SPHK1, whereas phosphatidylserine stimulated both type 1 and type 2 SPHK. Thus, SPHK2 is another member of a growing class of sphingolipid kinases that may have novel functions.

Sphingosine-1-phosphate (SPP)[1] is a bioactive sphingolipid metabolite that regulates diverse biological processes, acting both inside and outside cells (reviewed in Refs. 1 and 2). SPP plays important roles as a second messenger to regulate cell growth and survival (3, 4). Many external stimuli, particularly growth and survival factors, activate sphingosine kinase (SPHK), the enzyme that forms SPP from sphingosine. This rapidly growing list includes platelet-derived growth factor (3, 5–7), nerve growth factor (8, 9), vitamin D3 (10), muscarinic acetylcholine agonists (11), tumor necrosis factor-α (12), and cross-linking of the immunoglobulin receptors FcϵR1 (13) and FcγR1 (14). Intracellular SPP, in turn, mobilizes calcium from internal stores independently of inositol triphosphate (11, 15), as well as eliciting diverse signaling pathways leading to proliferation (16, 17) and suppression of apoptosis (4, 8, 17–19). Moreover, competitive inhibitors of SPHK block the formation of SPP and selectively inhibit calcium mobilization, cellular proliferation, and survival induced by these various stimuli (reviewed in Ref. 1). Thus, it has been suggested that the dynamic balance between levels of the sphingolipids metabolites, ceramide and SPP, and consequent regulation of opposing signaling pathways, is an important factor that determines the fate of cells (19). For example, stress stimuli increase ceramide levels leading to apoptosis, whereas survival factors stimulate SPHK leading to increased SPP levels, which suppress apoptosis (19). Moreover, the SPHK pathway, through the generation of SPP, is critically involved in mediating tumor necrosis factor-α-induced endothelial cell activation (12), and the ability of high density lipoproteins to inhibit cytokine-induced adhesion molecule expression has been correlated with its ability to reset this sphingolipid rheostat (12). This has important implications for the protective function of high density lipoproteins against the development of atherosclerosis and associated coronary heart disease. Recent data have also connected the sphingolipid rheostat to allergic responses (20).

Interest in SPP has accelerated recently with the discovery that it is a ligand of the G protein-coupled cell surface receptor EDG-1 (17, 21). This rapidly led to the identification of several other related receptors, named EDG-3, -5, -6, and -8, which are also specific SPP receptors (reviewed in Refs. 2 and 22). Sphinganine-1-phosphate, which is structurally similar to SPP and only lacks the trans double bond at the 4 position, but not lysophosphatidic acid or sphingosylphosphorylcholine, also binds to these receptors (23), demonstrating that EDG-1 belongs to a family of G protein-coupled receptors that bind SPP with high affinity and specificity (reviewed in Refs. 2 and 22). The EDG-1 family of receptors are differentially

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF245447 and AF245448.

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[1] The abbreviations used are: SPP, sphingosine-1-phosphate; SPHK, sphingosine kinase; EDG, endothelial differentiation gene; mSPHK, mouse SPHK; EST, expressed sequence tag; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; hSPHK, human SPHK; BSA, bovine serum albumin; kb, kilobase; DMS, *N,N*-dimethylsphingosine; *threo*-DHS, D,L-*threo*- dihydrosphingosine; TLC, thin layer chromatography; MES, 4-morpholineethanesulfonic acid.

expressed, mainly in the cardiovascular and nervous systems, and are coupled to a variety of G proteins and thus can regulate diverse signal transduction pathways culminating in pleiotropic responses depending on the cell type and relative expression of EDG receptors. Although the biological functions of the EDG-1 family of G protein-coupled receptors are not completely understood, recent studies suggest that binding of SPP to EDG-1 stimulates migration and chemotaxis (24, 25) and as a consequence may regulate angiogenesis (24, 26, 27). EDG-5 may play a role in cytoskeletal reorganization during neurite retraction, which is important for neuronal differentiation and development (23, 28).

Critical evaluation of the role of SPP requires cloning of the enzymes that regulate its metabolism. Recently, we purified rat kidney SPHK to apparent homogeneity (29) and subsequently cloned the first mammalian SPHK, designated mSPHK1 (30). Independently, two genes, termed LCB4 and LCB5, were also shown to code for SPHKs in *Saccharomyces cerevisiae* (31). Moreover, data base searches identified homologues of mSPHK1 in numerous widely disparate organisms, including worms, plants, and mammals, demonstrating that the enzyme is encoded by a member of a highly conserved gene family (30). Comparison of the predicted amino acid sequences of the known SPHK1s revealed five blocks of highly conserved amino acids (30). However, several lines of evidence indicate that there may be multiple mammalian SPHK isoforms. The finding that SPHK activity in platelets could be chromatographically fractionated into several forms with differing responses to detergents and inhibition by known SPHK inhibitors suggested the presence of multiple enzyme forms in human platelets (32). Moreover, homology searches against a comprehensive nonredundant data base revealed that several of the expressed sequence tags (dbEST) at NCBI had significant homology to conserved domains of mSPHK1 (30), yet they had substantial sequence differences. Thus, we embarked on an effort to clone other SPHK isoforms. We report here the cloning, functional characterization, and tissue distribution of a second type of mammalian SPHK (SPHK2) that has distinct sequence, properties, and tissue distribution.

EXPERIMENTAL PROCEDURES

Materials—SPP, sphingosine, and *N,N*-dimethylsphingosine were from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Poly-L-lysine and collagen were from Roche Molecular Biochemicals (Indianapolis, IN). Restriction enzymes were from New England Biolabs (Beverly, MA). Poly(A)$^+$ RNA blots of multiple mouse adult tissues were purchased from CLONTECH (Palo Alto, CA). LipofectAMINE PLUS and LipofectAMINE were from Life Technologies, Inc.

cDNA Cloning of Murine Sphingosine Kinase-2 (mSPHK2)—BLAST searches of the EST data base identified a mouse EST clone (GenBank™ accession number AA839233), which had significant homology to conserved domains of mSPHK1 (30) yet had substantial sequence differences. Using this EST, a second isoform of SPHK, denoted mSPHK2, was cloned by two different PCR approaches.

In the first, we used the method of PCR cloning from a mouse cDNA library (Stratagene). Approximately 1×10^6 phages were plated on twenty 150-mm plates, plaques were collected, and plasmids were isolated using standard procedures (33). An initial PCR reaction was carried out with a sequence specific primer (M-3–1, 5′-CCTGGGTC-CACCTGCGCCTGTATTGG) and the M13 reverse primer. The longest PCR products were gel-purified and used as the template for a second PCR, which contained a sequence-specific antisense primer (M-3–2, 5′-CCAGTCTTGGGGCAGTGGAGAGCC-3′) and the T3 primer. The final PCR products were subcloned by TOPO TA cloning (Invitrogen) and then sequenced. Platinum high fidelity DNA polymerase (Life Technologies, Inc.) was used for the PCR amplifications with the following cycling parameters: 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2 min with a final primer extension at 72 °C for 5 min.

In a second approach, 5′-RACE PCR was performed with the 5′-RACE System for rapid amplification of cDNA ends according to the manufacturer's protocol (Life Technologies, Inc.). Poly(A)$^+$ RNA was isolated from Swiss 3T3 fibroblasts using a Quick Prep mRNA purification kit (Amersham Pharmacia Biotech). First strand cDNA was synthesized at 42 °C for 50 min with 5 μg of Swiss 3T3 poly(A)$^+$ RNA using a target antisense primer designed from the sequence of AA839233 (m-GSP1, 5′-AGGTAGAGGCTTCTGG) and SuperScript II reverse transcriptase (Life Technologies, Inc.). Two consecutive PCR reactions using this cDNA as a template and LA *Taq* polymerase (TaKaRa) were carried out as follows: first PCR, 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and primer extension at 72 °C for 5 min with 5′-RACE Abridged Anchor Primer, 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG and the target-specific antisense primer m-GSP2, 5′-GCGATGGGTGAAAGCTGAGCTG; second PCR, same conditions except that the annealing temperature was 65 °C, with Abridged Universal Amplification Primer, 5′-GGCCACGCGTCGACTAGTAC and m-GSP3, 5′-AGTCTCCAGTCAGCTCTGGACC. PCR products were cloned into pCR2.1 and sequenced, and final PCR products were subcloned into pCR3.1 and pcDNA 3 expression vectors.

cDNA Cloning of Human Sphingosine Kinase-2 (hSPHK2)—Poly(A)$^+$ RNA from HEK293 cells was used for a 5′-RACE reaction. Target-specific antisense primers (h-GSP1, 5′-CCCACTCACTCAGGCT; h-GSP2, 5′-GAAGGACAGCCCAGCTTCAGAG; h-GSP3, 5′-ATTGACCAATAGAAGCAACC) were designed according to the sequence of a human EST clone (accession number AA295570). First strand cDNA was synthesized with 5 μg of HEK293 mRNA and h-GSP1. This cDNA was used as a template in an initial PCR reaction using 5′-RACE Abridged Anchor Primer and h-GSP2. Then, nested PCR was carried out using the Abridged Universal Amplification Primer and h-GSP3. The resulting PCR products were cloned and sequenced as described above.

Overexpression and Measurement of Activity of SPHK2—HEK293 cells (ATCC CRL-1573) and NIH 3T3 fibroblasts (ATCC CRL-1658) were cultured as described previously (34). HEK293 cells were seeded at 6×10^5/well in poly-L-lysine-coated 6-well plates. After 24 h, cells were transfected with 1 μg of vector alone or with vectors containing sphingosine kinase constructs and 6 μl of LipofectAMINE PLUS reagent plus 4 μl of LipofectAMINE reagent/well. 1–3 days after transfection, cells were harvested and lysed by freeze-thawing as described previously (30). In some experiments, cell lysates were fractionated into cytosol and membrane fractions by centrifugation at 100,000 $\times$ *g* for 60 min. SPHK activity was determined in the presence of sphingosine, prepared as a complex with 4 mg/ml BSA and [γ-^{32}P]ATP in kinase buffer (35) containing 200 mM KCl, unless indicated otherwise. ^{32}P-SPP was separated by TLC and quantified with a phosphoimager as described previously (30).

Lipid Extraction and Measurement of SPP—Cells were washed with phosphate-buffered saline and scraped in 1 ml of methanol containing a 2.5-μl conc. HCl. Lipids were extracted by adding 2 ml of chloroform, 1 M NaCl (1:1, v/v) and 100 μl of 3N NaOH, and the phases were separated. The basic aqueous phase containing SPP, and devoid of sphingosine, ceramide, and the majority of phospholipids, was transferred to a siliconized glass tube. The organic phase was re-extracted with 1 ml of methanol, 1 M NaCl (1:1, v/v) plus 50 μl of 3N NaOH, and the aqueous fractions were combined. Mass measurements of SPP in the aqueous phase and total phospholipids in the organic phase were carried out exactly as described (8, 36).

Northern Blotting Analysis—Poly(A)$^+$ RNA blots containing 2 μg of poly(A)$^+$ RNA/lane from multiple adult mouse and human tissues, and mouse embryos were purchased from CLONTECH. Blots were hybridized with the 1.2-kb *Pst*I fragment of mouse EST AA389187 (mSPHK1 probe), the 1.5-kb *Eco*RI fragment of pCR3.1-mSPHK2, the 0.3-kb *Pvu*II fragment of pCR3.1-hSPHK1, or the 0.6-kb *Eco*RV-*Sph*I fragment of human EST AA295570 (hSPHK2 probe), after gel-purification and labeling with [α-^{32}P]dCTP. Hybridization in ExpressHyb buffer (CLONTECH) at 65 °C overnight was carried out according to the manufacturer's protocol. Blots were reprobed with β-actin as a loading control (CLONTECH). Bands were quantified using an imaging analyzer (BAS2000, Fuji film).

RESULTS AND DISCUSSION

Cloning of Type 2 Sphingosine Kinase

Blast searches of the EST data base identified several ESTs that displayed significant homology to our recently cloned mSPHK1 sequence (30). Specific primers were designed from

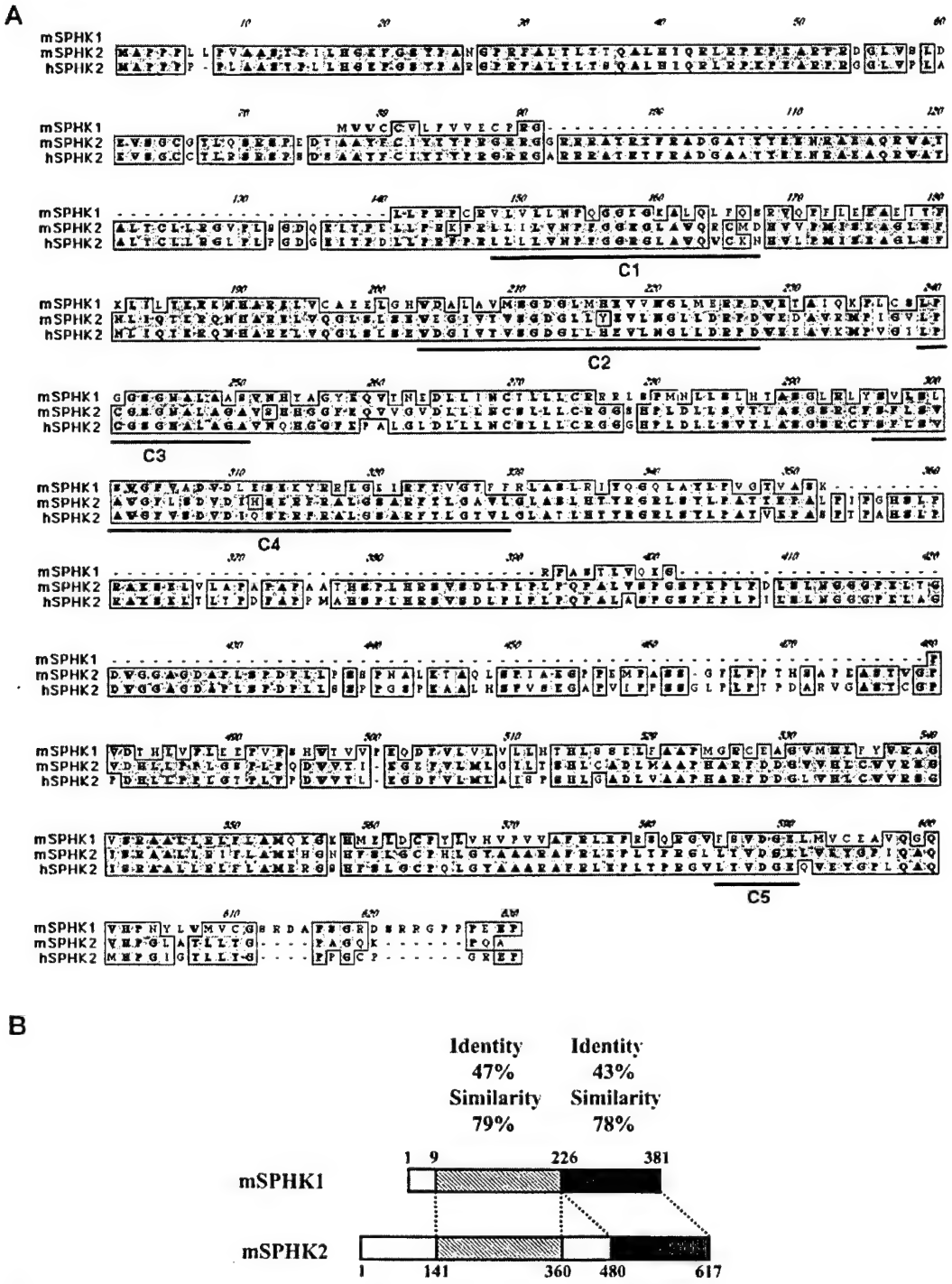

FIG. 1. **Predicted amino acid sequences of murine and human type 2 SPHK.** *A*, ClustalW alignment of the predicted amino acid sequences of mSPHK2 and hSPHK2. Identical and conserved amino acid substitutions are shaded *dark* and *light gray*, respectively. The *dashes* represent gaps in sequences, and *numbers* on the right refer to the amino acid sequence of mSPHK2. The conserved domains (C1–C5) are indicated by *lines*. *B*, schematic representation of conserved regions of SPHK1 and SPHK2. The primary sequence of mSPHK2 is compared with that of mSPHK1.

the sequences of these ESTs and were used to clone a new type of mouse and human SPHK (named mSPHK2 and hSPHK2) by the approaches of PCR cloning from a mouse brain cDNA library and 5′-RACE PCR.

ClustalW alignment of the amino acid sequences of mSPHK2 and hSPHK2 is shown in Fig. 1*A*. The open reading frames of mSPHK2 and hSPHK2 encode polypeptides of 617 and 618 amino acids, respectively, with 83% identity and 90% similarity. Five highly conserved regions (C1–C5), identified previously in SPHK1s (30), are also present in both type 2 kinases.

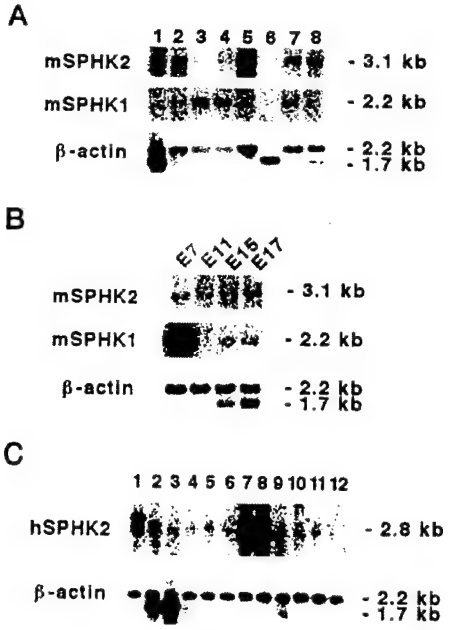

FIG. 2. **Tissue-specific expression of type 1 and type 2 SPHK.** *A*, mSPHK2 (*upper panel*) and mSPHK1a (*middle panel*) probes were end-labeled and hybridized to poly(A)$^+$ RNA blots from the indicated mouse tissues as described under "Experimental Procedures." *Lane 1*, heart; *lane 2*, brain; *lane 3*, spleen; *lane 4*, lung; *lane 5*, liver; *lane 6*, skeletal muscle; *lane 7*, kidney; *lane 8*, testis. A β-actin probe (*lower panel*) was used as a loading control. *B*, expression of mSPHK1a and mSPHK2 during mouse embryonic development. Poly(A)$^+$ RNA blots from embryonic days (*E*) 7, 11, 15, and 17 mouse embryos were probed as in *A*. *C*, tissue-specific expression of hSPHK2. *Lane 1*, brain; *lane 2*, heart; *lane 3*, skeletal muscle; *lane 4*, colon; *lane 5*, thymus; *lane 6*, spleen; *lane 7*, kidney; *lane 8*, liver; *lane 9*, small intestine; *lane 10*, placenta; *lane 11*, lung; *lane 12*, leukocyte.

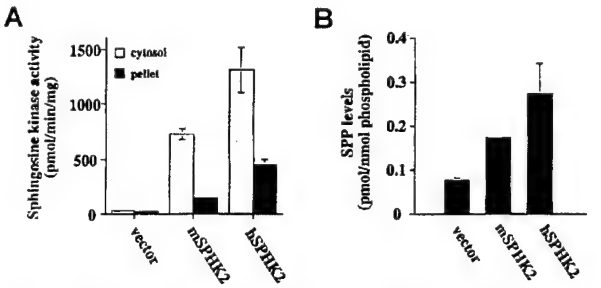

FIG. 3. **Enzymatic activity of recombinant SPHK2.** *A*, HEK293 cells were transiently transfected with empty vector or with mSPHK2 or hSPHK2 expression vectors. After 24 h, SPHK activity was measured in cytosol (*open bars*) and particulate fractions (*filled bars*). The data are mean ± S.D. Parental and vector-transfected cells had basal SPHK activities of 26 and 37 pmol/min/mg, respectively. *B*, changes in mass levels of SPP after transfection with SPHK2. Mass levels of SPP in HEK293 cells transfected with empty vector, with mSPHK2 (*filled bars*), or with hSPHK2 were measured as described under "Experimental Procedures." The data are expressed as pmol/nmol phospholipid.

Interestingly, the invariant GGKGK positively charged motif in the C1 domain of SPHK1 is modified to GGRGL in SPHK2, suggesting that it may not be part of the ATP binding site as previously proposed (30). A motif search also revealed that a region beginning just before the conserved C1 domains of mSPHK2 and hSPHK2 (amino acid 147–284) also has homology to the diacylglycerol kinase catalytic site.

Compared with SPHK1, both SPHK2s encode much larger proteins containing 236 additional amino acids (Fig. 1*B*). Moreover, their sequences diverge considerably from SPHK1 in the center and at the amino termini. However, after amino acid 140 of mSPHK2, the sequences of type 1 and type 2 mSPHK have a large degree of similarity. These sequences (amino acids 9–226 for mSPHK1 and 141–360 for mSPHK2), which encompass domains C1–C4, have 47% identity and 79% similarity (Fig. 1*B*). In the carboxyl-terminal portion of the proteins there are also large homologous regions, which include the C5 domain, from amino acids 227–381 for mSPHK1 and 480–617 for mSPHK2, with 43% identity and 78% similarity (Fig. 1*B*). The overall divergence suggests that SPHK2 probably did not arise as a simple gene duplication event.

Tissue Distribution of Sphingosine Kinase Type 2

The tissue distribution of SPHK2 mRNA expression in adult mouse was compared with that of SPHK1 by Northern blotting (Fig. 2*A*). In most tissues, including adult liver, heart, kidney, testis, and brain, a predominant 3.1-kb SPHK2 mRNA species was detected, indicating ubiquitous expression. However, the level of expression was markedly variable and was highest in adult liver and heart and barely detectable in the skeletal muscle and spleen (Fig. 2*A*). In contrast, the expression pattern of mSPHK1 is quite different, with highest mRNA expression of a 2.2-kb transcript in adult lung, spleen, and liver, although expression in liver does not predominate as with mSPHK2. mSPHK1 and mSPHK2 were both temporally and differentially expressed during embryonic development. mSPHK1 was expressed highly at mouse embryonic day 7 and decreased dramatically after embryonic day 11 (Fig. 2*B*). In contrast, at embryonic day 7, mSPHK2 expression was much lower than mSPHK1 and gradually increased up to embryonic day 17. The hSPHK2 2.8-kb mRNA transcript was mainly expressed in adult kidney, liver, and brain, with much lower expression in other tissues (Fig. 2*C*). Interestingly, expression of SPHK2 in human kidney is very high and relatively much lower in the mouse kidney, whereas the opposite pattern holds for the liver.

Activity of Recombinant Sphingosine Kinase Type 2

To investigate whether mSPHK2 and hSPHK2 encode *bona fide* SPHKs, HEK293 cells were transiently transfected with expression vectors containing the corresponding cDNAs. Because previous studies have indicated that SPHK might be present in cells in both soluble and membrane-associated forms (3, 32, 37–39), recombinant SPHK2 activity was measured both in cytosol and in membrane fractions of transfected cells. As described previously (30), untreated or vector-transfected HEK293 cells have low levels of SPHK activity (Fig. 3*A*). 24 h after transfection with mSPHK2 or hSPHK2, *in vitro* SPHK activity was increased by 20- and 35-fold, respectively, and then decreased thereafter (Fig. 3*A*). In contrast, SPHK activity in cells transfected with mSPHK1 was much higher, 610-fold greater than basal levels 24 h after transfection and remained at this level for at least 3 more days (data not shown). As in HEK293 cells, transfection of NIH 3T3 fibroblasts with mSPHK1 resulted in much higher SPHK activity than with mSPHK2. We previously found that, similar to untransfected cells, the majority of SPHK activity in cells transfected with mSPHK1 was cytosolic (30). Similarly, in cells transfected with either mSPHK2 or hSPHK2, 17 and 26%, respectively, of the SPHK activity was membrane-associated (Fig. 3*A*), although Kyte-Doolittle hydropathy plots did not suggest the presence of hydrophobic membrane-spanning domains.

Transfection of HEK293 cells with mSPHK2 and hSPHK2 also resulted in 2.2- and 3.3-fold increases, respectively, in

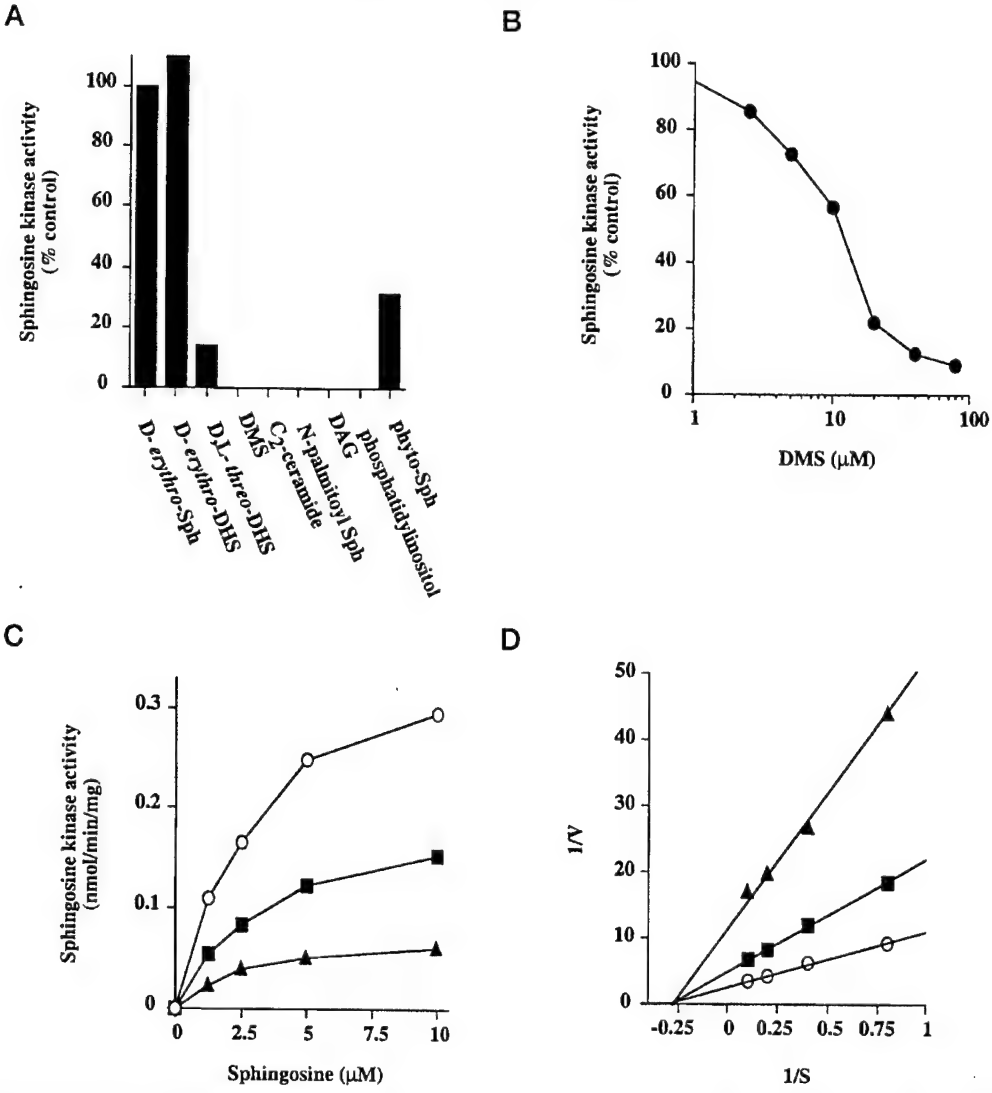

FIG. 4. **Substrate specificity of mSPHK2.** *A*, SPHK-dependent phosphorylation of various sphingosine analogs or other lipids (50 μM) was measured in cytosol from HEK293 cells transfected with mSPHK2. Data are expressed as percentage of phosphorylation of D-*erythro*-Sph. *B–D*, noncompetitive inhibition of recombinant SPHK2 by *N,N*-dimethylsphingosine. *B*, dose-dependent inhibition of mSPHK2 by DMS. SPHK activity in HEK293 cell lysates after transfection as in *A* was measured with 10 μM D-*erythro*-sphingosine in the presence of increasing concentrations of DMS. *C*, kinetic analysis of DMS inhibition. SPHK activity was measured with varying concentrations of D-*erythro*-sphingosine in the absence (*open circles*) or presence of 10 (*filled squares*) or 20 μM DMS (*filled triangles*). *D*, Lineweaver-Burk plots. The K_m value for D-*erythro*-sphingosine was 3.4 μM. The K_i value for DMS was 12 μM.

SPP, the product formed by SPHK (Fig. 3*B*), in agreement with previous studies of sphingolipid metabolite levels after transfection with mSPHK1, which showed a lack of correlation of fold increases in SPP levels and *in vitro* SPHK enzyme activity (30, 34).

Characteristics of Recombinant mSPHK2

Substrate Specificity—Although SPHK2 is highly homologous to SPHK1, there are substantial sequence differences. Therefore, it was of interest to compare their enzymatic properties. Typical Michaelis-Menten kinetics were observed for recombinant SPHK2 (data not shown). The K_m for D-*erythro*-sphingosine as substrate is 3.4 μM, almost identical to the K_m previously found for SPHK1 (29). Although the naturally occurring D-*erythro*-sphingosine isomer was the best substrate for SPHK1 (30), D-*erythro*-dihydrosphingosine was a better substrate for SPHK2 than D-*erythro*-sphingosine (Fig. 4*A*). Moreover, although D,L-*threo*-dihydrosphingosine and phytosphingosine were not phosphorylated at all by SPHK1, they were significantly phosphorylated by SPHK2, albeit much less efficiently than sphingosine. Like SPHK1, other lipids including *N,N*-dimethylsphingosine (DMS), C_2- or C16-ceramide, diacylglycerol, or phosphatidylinositol, were not phosphorylated by SPHK2 (Fig. 4*A*), suggesting high specificity for the sphingoid base.

DMS and *threo*-DHS have previously been shown to be potent competitive inhibitors of SPHK1 (40) and have been used to block increases in intracellular SPP levels resulting from various physiological stimuli (3, 4, 8, 11, 13, 14, 41). However, because *threo*-DHS is a substrate for SPHK2, it is not useful as a tool to investigate the role of SPHK2/SPP signaling. Thus, it was important to characterize the inhibitory potential of the nonsubstrate DMS on SPHK2. Surprisingly, we found that

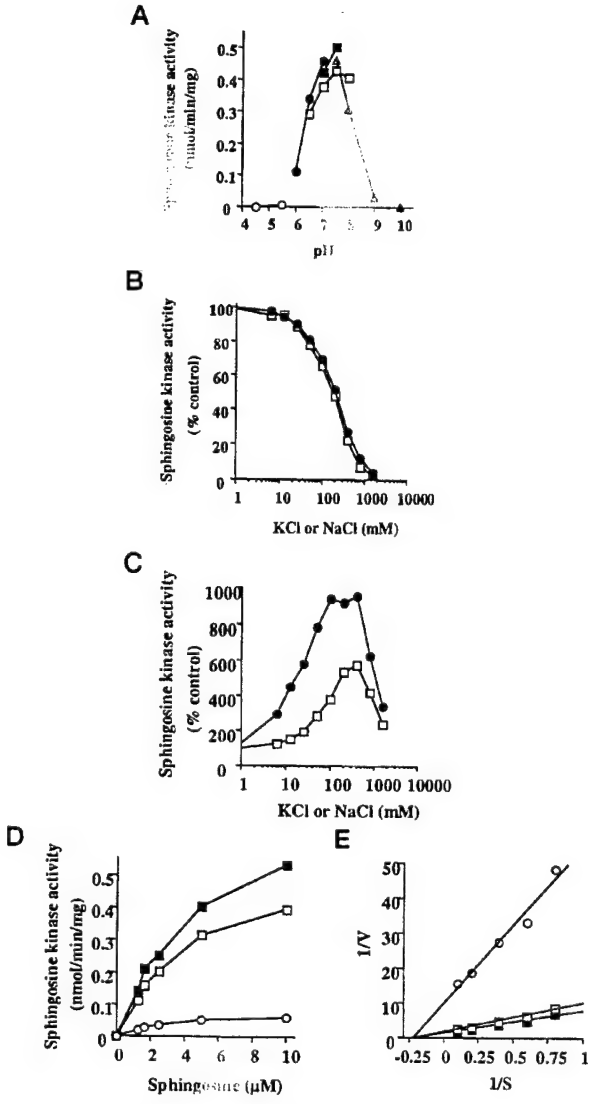

FIG. 5. **pH dependence and salt effects on mSPHK2.** *A*, cytosolic SPHK2 activity in transfected HEK293 cells was measured in kinase buffer with the pH adjusted using the following buffers: 200 mM sodium acetate (pH 4.5–5.5, *open circles*); 200 mM MES (pH 6–7, *filled circles*); 200 mM potassium phosphate (pH 6.5–8, *open squares*); 200 mM HEPES (pH 7–7.5, *filled squares*); 200 mM Tris-HCl (pH 7.5–9, *open triangles*); and 200 mM borate (pH 10, *filled triangle*). *B–E*, salts stimulate SPHK2 but inhibit SPHK1. *B* and *C*, SPHK activity in HEK293 cell lysates was measured 24 h after transfection with mSPHK1 (*B*) or mSPHK2 (*C*) in the absence or presence of increasing concentrations of NaCl (*open squares*) or KCl (*filled circles*). *D*, kinetic analysis of SPHK2 activation by KCl. mSPHK2 activity was measured with varying concentrations of D-*erythro*-sphingosine in the absence (*open circles*) or presence of 50 (*open squares*) or 200 mM KCl (*filled circles*). *E*, Lineweaver-Burk plots of data from *D*. The K_m value is not affected by the presence of KCl. V_{max} values were 0.1, 0.3, and 1 (nmol/min/mg) in the presence of 0, 50, and 200 mM KCl, respectively.

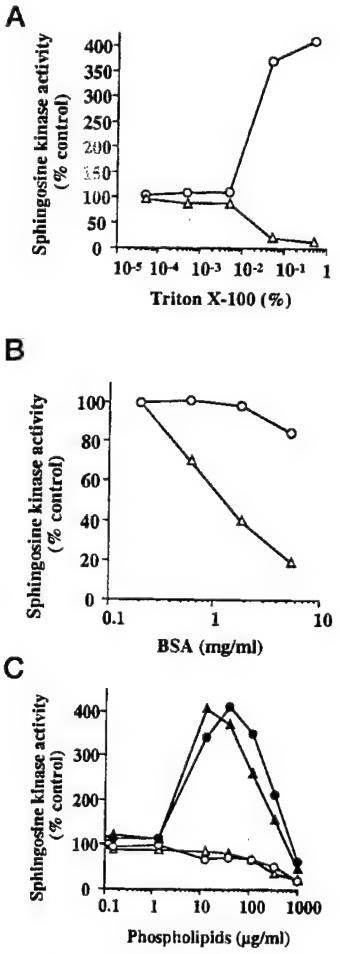

FIG. 6. **Triton X-100 and BSA, but not phosphatidylserine, have differential effects on activity of SPHK1 and SPHK2.** HEK293 cells were transfected with mSPHK1 (*circles*) or mSPHK2 (*triangles*) and the activities of each in cell lysates were measured after 24 h in the presence of the indicated concentrations of Triton X-100 (*A*), BSA (*B*), or phosphatidylserine (*filled symbols*) or phosphatidylcholine (*open symbols*) (*C*). Data are expressed as percentage of control activity measured without any additions.

although DMS was also a potent inhibitor of SPHK2 (Fig. 4*B*), it acted in a noncompetitive manner (Fig. 4, *C* and *D*). The K_i for DMS with SPHK2 was 12 μM, slightly higher than the K_i of 4 μM with SPHK1, suggesting that it can be used to inhibit both types of SPHK.

mSPHK2 had highest enzymatic activity in the neutral pH range from 6.5 to 8 with optimal activity at pH 7.5 (Fig. 5*A*), a pH dependence similar to that of SPHK1 (data not shown). The activity decreased markedly at pH values below and above this range.

Effects of KCl and NaCl—Most of the SPHK activity in human platelets is membrane-associated and extractable with 1 M NaCl (32). Furthermore, the salt extractable SPHK from platelets has different properties than the cytosolic enzyme. It was thus of interest to determine the effect of high salt concentrations on recombinant SPHK1 and SPHK2. Interestingly, we found that high ionic strength had completely opposite effects on their activities. SPHK1 was markedly inhibited by either NaCl and KCl, with each causing 50% inhibition at a concentration of 200 mM (Fig. 5*B*). In contrast, SPHK2 activity was dramatically stimulated by increasing the salt concentration, with a maximal effect at a concentration of 400 mM, although KCl was much more effective than NaCl. However, above this concentration, SPHK2 activity decreased sharply although remaining elevated even at 1 M salt (Fig. 5*C*). Kinetic analysis of mSPHK2 in the presence and absence of high concentrations of salt indicated that the K_m for sphingosine was unaltered, whereas the V_{max} was increased (Fig. 5, *D* and *E*). The physi-

ological significance of these observations remains to be determined but it could be related to different subcellular localizations of the two types of SPHK.

Substrate Presentation—Because sphingolipids are highly lipophilic, in *in vitro* SPHK assays, sphingosine is usually presented in micellar form with Triton X-100 or as a complex with BSA (42, 43). Furthermore, detergents such as Triton X-100 have been shown to stimulate the activity of SPHK in rat brain extracts (37) and the enzyme from rat kidney (29), and we previously found that the stability of rat kidney SPHK was increased in the presence of certain detergents (29). However, when the effect of increasing concentrations of Triton X-100 on the activities of SPHK1 and SPHK2 were compared, some unexpected results were found. Concentrations of detergent up to 0.005% had no effect, but at higher concentrations, SPHK2 activity was inhibited and SPHK1 activity was markedly stimulated (Fig. 6*A*). At a concentration of Triton X-100 of 0.5%, SPHK1 activity was increased by more than 4-fold, whereas SPHK2 was almost completely inhibited. Thus, Triton X-100 could be used to differentially determine SPHK1 and SPHK2 activities in tissues or cells that express both types.

Interestingly, increasing the BSA concentration from our usual SPHK assay conditions with sphingosine-BSA complex as substrate, *i.e.* 0.2 mg/ml BSA, caused a concentration-dependent inhibition of SPHK2 activity without affecting SPHK1 activity (Fig. 6*B*). Therefore, when measuring SPHK activity in cell or tissue extracts, the method of substrate presentation, whether in mixed micelles or in BSA complexes, must be carefully optimized because the differential effects of Triton X-100 and BSA on activity could yield different results depending on the relative expression of the two types of SPHK.

Effects of Phospholipids—Acidic phospholipids, particularly phosphatidylserine, phosphatidic acid, and phosphatidylinositol, and cardiolipin to a lesser extent, induced a dose-dependent increase in SPHK activity in Swiss 3T3 fibroblast lysates, whereas neutral phospholipids had no effect (42). In agreement, the enzymatic activity of recombinant SPHK1 and SPHK2 was stimulated by phosphatidylserine; the activity of both was maximally increased 4-fold at a concentration of 40 μg/ml (Fig. 6*C*) and inhibited by higher concentrations in a dose-dependent manner. These effects of phosphatidylserine appeared to be specific because other phospholipids, including phosphatidylcholine, had no effect on the enzyme activity. In contrast, the activities of the three major forms of SPHK in human platelets were not affected by phosphatidylserine (32).

The mechanism by which phosphatidylserine enhances the enzymatic activity of SPHK is not yet understood. One possibility is that phosphatidylserine possesses unique membrane-structuring properties, which better present the substrate, sphingosine. A second possibility is that SPHK contains determinants that specifically recognize the structure of the serine headgroup and that these determinants may only become exposed upon interaction of SPHK with membranes. In this regard, the molecular basis for the remarkable specificity of protein kinase C for phosphatidylserine has been the subject of much debate. However, recent data reveal that lipid structure and not membrane structure is the major determinant in the regulation of protein kinase C by phosphatidylserine (44).

Concluding Remarks

The presence of multiple ESTs in the data base with significant homologies to SPHK1 as well as the identification of several genes in *S. cerevisiae* encoding different SPHKs (31) suggested that there may be a large and important SPHK gene family. In this study, we have cloned and characterized a second type of SPHK that has some unique properties. Although SPHK2 has a high degree of homology to SPHK1, especially in the previously identified conserved domains identified in type 1 SPHKs (30), it is much larger (65.2 and 65.6 kDa for hSPHK2 and mSPHK2, respectively, *versus* 42.4 kDa for mSPHK1) and contains an additional 236 amino acids. Furthermore, its differential tissue expression, temporal developmental expression, cellular localization, and kinetic properties in response to increasing ionic strength and detergents are completely different from SPHK1, suggesting that it most likely has a different function and regulates levels of SPP in a different manner than SPHK1, which is known to play a prominent role in regulating cell growth and survival. Thus, type 2 SPHK might be involved in regulation of some of the numerous biological responses attributed to SPP, such as angiogenesis and allergic responses.

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Functional characterization of human sphingosine kinase-1

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Abstract **Sphingosine kinase catalyzes the phosphorylation of sphingosine to form sphingosine 1-phosphate (SPP), a novel lipid mediator with both intra- and extracellular functions. Based on sequence identity to murine sphingosine kinase (mSPHK1a), we cloned and characterized the first human sphingosine kinase (hSPHK1). The open reading frame of hSPHK1 encodes a 384 amino acid protein with 85% identity and 92% similarity to mSPHK1a at the amino acid level. Similar to mSPHK1a, when HEK293 cells were transfected with hSPHK1, there were marked increases in sphingosine kinase activity resulting in elevated SPP levels. hSPHK1 also specifically phosphorylated D-*erythro*-sphingosine and to a lesser extent sphinganine, but not other lipids, such as D,L-*threo*-dihydrosphingosine, *N,N*-dimethylsphingosine, diacylglycerol, ceramide, or phosphatidylinositol. Northern analysis revealed that hSPHK1 was widely expressed with highest levels in adult liver, kidney, heart and skeletal muscle. Thus, hSPHK1 belongs to a highly conserved unique lipid kinase family that regulates diverse biological functions.**

Key words: Human sphingosine kinase; Sphingosine 1-phosphate

1. Introduction

The metabolic product of sphingosine kinase (SPHK), sphingosine 1-phosphate (SPP), is a lipid signaling molecule that acts both intra- and extracellularly to affect many biological processes. These include mitogenesis [1,2], apoptosis [3], atherosclerosis [4] and inflammatory responses [5,6]. Specific members of the EDG-1 family of G protein-coupled receptors bind SPP (reviewed in [7,8]) and modulate chemotaxis [9,10], angiogenesis [10–12], neurite retraction and cell rounding [13]. Because SPP levels are mainly regulated by the activity of SPHK, cloning and characterization of this enzyme are important for understanding its role in normal and pathological processes. Previously, we purified SPHK to homogeneity from rat kidneys [14] and subsequently identified mouse cDNAs encoding two forms of SPHK, designated mSPHK1a and mSPHK1b, whose predicted proteins differ by only 10 amino acids at their N-terminus [15]. The corresponding mRNAs may arise by alternative splicing. In this study, sequence homologies to the mSPHK1a cDNAs were used to identify and clone the first human homologue, hSPHK1. hSPHK1 is ubiquitously expressed in adult tissues with highest levels in liver, kidney, lung and skeletal muscle. Our results suggest that hSPHK1 belongs to a family of highly conserved enzymes which differ from other known lipid kinases.

2. Materials and methods

2.1. Materials

SPP, sphingosine, and *N,N*-dimethylsphingosine (DMS) were from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Poly-L-lysine was from Boehringer Mannheim (Indianapolis, IN). Alkaline phosphatase from bovine intestinal mucosa, type VII-NT, was from Sigma (St. Louis, MO). Restriction enzymes were from New England Biolabs (Beverly, MA). Lipofectamine Plus was from Life Technologies (Gaithersburg, MD).

2.2. Human sphingosine kinase cDNA cloning

BLAST searches using mSPHK1a sequences identified an EST clone (AA026479) which contained sequences homologous to several conserved domains of mSPHK [15]. To obtain a full-length cDNA, the 5'-end of hSPHK1 was extended by rapid amplification of cDNA ends/polymerase chain reaction (RACE-PCR; Life Technologies). First, cDNA was synthesized from HEK293 poly(A)$^+$ RNA with a gene-specific antisense primer hspk1-GSP1 (5'-ACCATTGTCCAGTGAG). Then two consecutive PCR reactions using LA Taq (TaKaRa) were performed. First PCR: 5'RACE Abridged Anchor Primer and the antisense primer hspk1-GSP2 (5'-TTCCTACAGGGAGGTAGGCC) at 94°C for 2 min followed by 30 cycles of amplification (94°C for 1 min, 55°C for 1 min, 72°C for 2 min) and primer extension at 72°C for 5 min. Second PCR: Abridged Universal Amplification Primer and the antisense primer hspk1-GSP3 (5'-GGCTGCCAGACGCAGGAAGG) using a program similar to the first PCR but with annealing at 65°C. The PCR products were cloned into pCR 2.1 (TA Cloning, Invitrogen) and sequences confirmed by automated sequencing. To make expression constructs, a primer set was designed as follows: sense primer containing a Kozak sequence and ATG start codon, sphk1-GSP4 (5'-GCCACCATGGATCCAGCGGGCGGCCCC); antisense primer, sphk1-GSP5 (5'-TCATAAGGGCTCTTCTGGCGGTGGCATCTG). The PCR reaction was performed using human fetus Marathon-Ready cDNA (Clontech) as template with the above primers, and the amplification product was subcloned into pCR3.1 (Eukaryotic TA Cloning, Invitrogen). In addition, hSPHK1 was tagged at the N-terminus by subcloning into a pcDNA-c-myc vector [2] using high fidelity taq polymerase (Pfu, Stratagene). hSPHK1 accession number is AF238083.

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Abbreviations: BSA, bovine serum albumin; DMS, *N,N*-dimethylsphingosine; DHS, D,L-*threo*-dihydrosphingosine; SPHK, sphingosine kinase; SPP, sphingosine 1-phosphate; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends

PII: S0014-5793(00)01510-6

2.3. Cell culture and expression of sphingosine kinase

Human embryonic kidney cells (HEK293, ATCC CRL-1573) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine supplemented with 10% fetal bovine serum [15]. Cells were transfected with either pcDNA3.1 or pCR3.1 containing hSPHK1 using Lipofectamine Plus according to the manufacturer's protocol. Transfection efficiencies were typically about 40%.

2.4. Measurement of sphingosine kinase activity

Cytosolic sphingosine kinase activity was determined with 50 μM sphingosine, dissolved in 5% Triton X-100 (final concentration 0.25%) and [γ-^{32}P]ATP (10 μCi, 1 mM) containing $MgCl_2$ (10 mM) as previously described [15]. In some experiments, sphingosine was added as a complex with bovine serum albumin (BSA) as previously described [15]. Specific activity is expressed as pmol SPP formed per min per mg protein.

2.5. Lipid extraction and measurement of SPP, sphingosine, and ceramide

Cells were washed with phosphate buffered saline and scraped in 1 ml of methanol containing 2.5 μl concentrated HCl. Lipids were extracted by adding 2 ml chloroform/1 M NaCl (1:1, v/v) and 100 μl 3 N NaOH and phases were separated. The basic aqueous phase containing SPP, and devoid of sphingosine, ceramide, and the majority of phospholipids, was transferred to a siliconized glass tube. The organic phases were re-extracted with 1 ml methanol/1 M NaCl (1:1, v/v) plus 50 μl 3 N NaOH, and the aqueous fractions combined. Mass measurements of SPP in the aqueous phase were carried out as previously described [16]. Sphingosine and ceramide in the organic phase were determined by enzymatic methods using sphingosine kinase and diacylglycerol kinase, respectively [17]. Total phospholipids present in lipid extracts were also quantified [17].

2.6. Northern blotting analysis

Poly(A)$^+$ RNA blots containing 2 μg of poly(A)$^+$ RNA per lane from multiple adult human tissues (Clontech) were hybridized with the 0.6 kb *Eco*RV/*Sph*I fragment of pCR3.1-hSPHK1, which was gel-purified and labeled with [^{32}P]dCTP by random priming. Hybridization in ExpressHyb buffer (Clontech) was carried out at 65°C overnight according to the manufacturer's protocol. Blots were reprobed with a human β-actin control probe (Clontech). Bands were quantified using a Molecular Dynamics Phosphoimager.

3. Results and discussion

3.1. Cloning of hSPHK1

BLAST searches of the EST database identified a human homologue of murine SPHK, EST AA026479, with similarity to the 3′ end of mSPHK1a. This sequence was used to design specific primers and 5′ RACE was performed on mRNA extracted from HEK293 cells to obtain the full-length cDNA of hSPHK1. The open reading frame encodes a protein with 384 amino acids, and 85% identity and 92% similarity to mSPHK1a at the amino acid level (Fig. 1). We previously found by sequence alignment that SPHKs from mouse, yeast and *Caenorhabditis elegans* share several conserved blocks of amino acids [15]. Similarly, hSPHK1 contains these conserved regions (C1–C5, Fig. 1), including the invariant positively charged motif, GGKGK, in the C1 domain, which may be part of the ATP binding site of this novel class of lipid kinases.

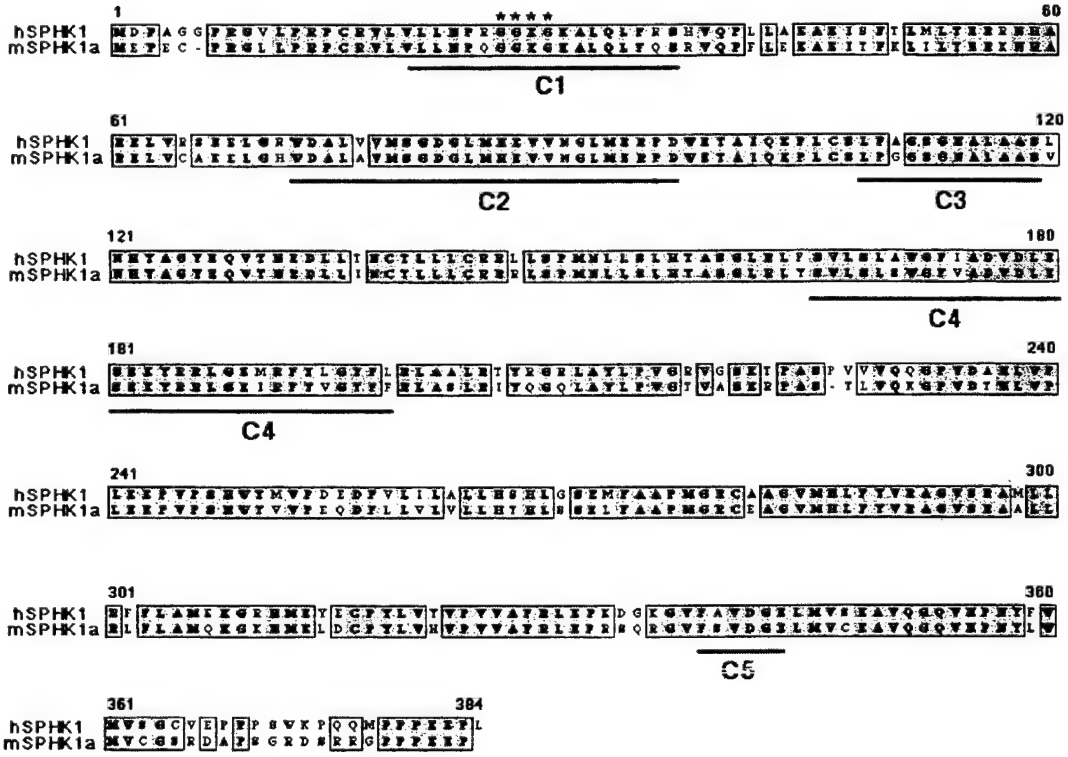

Fig. 1. Predicted amino acid sequence of hSPHK1 and alignment of the conserved domains. ClustalW alignment of SPHKs from mouse and human. Identical and conserved amino acid substitutions are shaded dark and light gray, respectively. The conserved domains (C1–C5) are indicated by lines and the invariant positively charged motif GGKGK by asterisks.

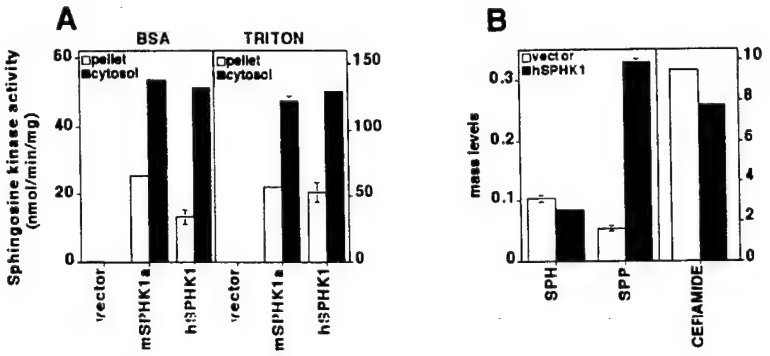

Fig. 2. Activity of hSPHK1 expressed in HEK293 cells. A: HEK293 cells were transiently transfected with empty vector or vector containing either mSPHK1a or hSPHK1. SPHK activity was measured in cytosol (filled bars) and particulate pellet (open bars) 24 h after transfection using sphingosine–BSA complexes or sphingosine–Triton X-100 micelles as substrate as indicated. SPHK activity in vector transfected cells was 84 ± 2 and 134 ± 27 pmol/min/mg using sphingosine–BSA complexes or sphingosine–Triton X-100 micelles as substrate, respectively. Data are means ± S.D. and are representative of two independent experiments performed in triplicate. B: Changes in mass levels of SPP, sphingosine, and ceramide. Mass levels of SPP, sphingosine and ceramide in cells transfected with empty vector (open bars) or vector containing hSPHK1 (filled bars) were measured after 24 h. Data are expressed as pmol/nmol phospholipid and are means ± S.D. of triplicate determinations.

3.2. *hSPHK1 encodes a functional sphingosine kinase*

HEK293 cells were transfected with expression vectors containing hSPHK1 to determine whether it encodes a bona fide SPHK. Modest levels of endogenous SPHK activity were detected in cells transfected with an empty vector (Fig. 2A). Twenty-four hours after transfection with pcDNA3.1-hSPHK1, the SPHK activity increased approximately 600-fold and remained at this level for at least 2 days. For comparison, a similar increase in activity was observed after transfection with mSPHK1a (Fig. 2A). Similar results were obtained when cells were transfected with hSPHK1 in pCR3.1. In agreement with previous results with mSPHK1a [15], hSPHK1 was stimulated by Triton X-100. Both membrane-associated and cytosolic SPHK activity have been described in mammalian tissues and cell lines [1,18–21]. In cells transfected with hSPHK1, approximately 70% of the SPHK activity was found in the cytosol and only about 30% was membrane-associated (Fig. 2A). Similarly, we previously found that the majority of mSPHK1a activity was also expressed in the cytosol [2,15]. Kyte–Doolittle hydropathy plots did not suggest the presence of any potential hydrophobic membrane spanning domains in the primary structure of hSPHK1.

Transfection of HEK293 cells with hSPHK1 also resulted in changes in levels of sphingolipid metabolites (Fig. 2B). Mass levels of SPP increased 5.7-fold compared to cells transfected with vector alone, with a 18% decrease in levels of both sphingosine and ceramide. However, because intracellular ceramide pools are much larger than sphingosine pools, the absolute decrease of ceramide was greater than the decrease in sphingosine mass. These results suggest that transfected hSPHK1 is active in intact cells, and that kinase overexpression can alter the intracellular balance of sphingolipid metabolites.

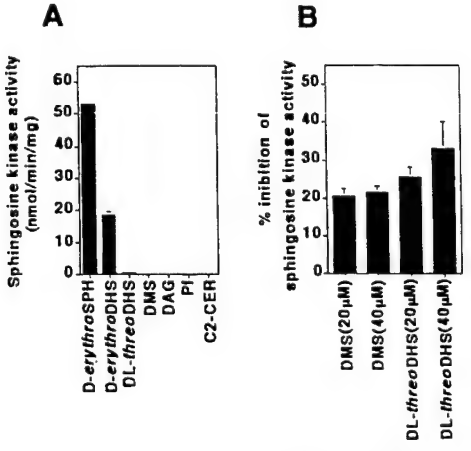

Fig. 3. A: Substrate specificity of hSPHK1. HEK293 cells were transfected with hSPHK1 and SPHK-dependent phosphorylation of various sphingosine analogs or other lipids (50 μM) was measured using cell lysates as enzyme source. DAG, diacylglycerol; PI, phosphatidylinositol; C2-CER, *N*-acetyl-sphingosine. B: DMS and DHS are inhibitors of hSPHK1. SPHK activity in HEK293 cell lysates 24 h after transfection with hSPHK1 was measured with 10 μM SPP in the absence or presence of 20 μM and 40 μM DMS or DHS. Data are means ± S.D. of triplicate determinations and are expressed as percent inhibition.

3.3. *Substrate specificity of hSPHK1*

The naturally occurring D-(+)-*erythro-trans*-isomer of sphingosine and *erythro*-dihydrosphingosine (sphinganine) were the best substrates for hSPHK1 (Fig. 3A). However, similar to the specificity of mSPHK1a [15], sphingosine was more efficiently phosphorylated than sphinganine. Moreover, other sphingo-

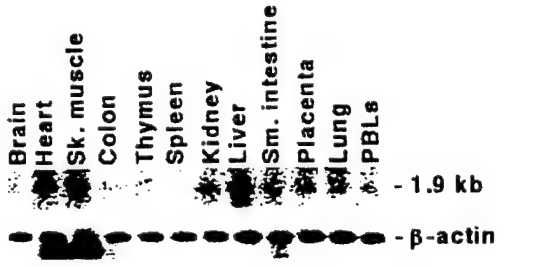

Fig. 4. Tissue-specific expression of hSPHK1 by Northern blot analysis. Top panel: A hSPHK1 probe was hybridized to a poly(A)$^+$ RNA blot with the human tissues indicated at the top of each lane as described in Section 2. Bottom panel: A β-actin probe was used to reprobe the blot.

lipids, including D,L-*threo*-dihydrosphingosine (DHS) and C2-ceramide, as well as diacylglycerol and phosphatidylinositol were not substrates (Fig. 3A). With D-*erythro*-sphingosine as substrate, half-maximal velocity was found at 5 μM, in excellent agreement with K_m values previously determined with rat kidney SPHK [14] and recombinant mSPHK1a [15]. DMS and DHS have previously been used to inhibit SPHK and block increases in SPP induced by various physiological stimuli [1,3,22]. Both of these sphingolipids also inhibited hSPHK1 and similar to their inhibitory effects on mSPHK1 [15], DHS was slightly more potent than DMS (Fig. 3B).

3.4. Tissue distribution of hSPHK1 expression

The tissue distribution of SPHK1 mRNA expression in adult human tissues was analyzed by Northern blotting (Fig. 4). In most tissues, including adult brain, heart, spleen, lung, kidney, and testis, a predominant 1.9 kb mRNA species was detected. Expression was highest in adult liver, heart and skeletal muscle. In comparison, we previously showed that mSPHK1a expression is greatest in mouse spleen, lung, kidney, testis and heart, with much lower expression in skeletal muscle [15].

In summary, hSPHK1 is the human homolog of mSPHK1. Based on EST sequences, hSPHK1 has been localized on chromosome 17q25.2 at the marker stSG28540 (D17S785-D17S836 Reference Interval, UniGene cluster Hs. 68061, URL: http://www.ncbi.nlm.nih.gov/unigene/clust.cgi?org=hs and cid=68061). hSPHK1 belongs to a conserved family of genes that is distinct from other known lipid kinases. Molecular cloning and characterization of members of the SPHK family should help to clarify their potential roles in various human diseases as their product, SPP, has been implicated as an important regulatory component of biological processes including growth, survival, allergy, chemotaxis, and angiogenesis.

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Sphingosine-1-phosphate Inhibits Motility of Human Breast Cancer Cells Independently of Cell Surface Receptors[1]

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ABSTRACT

Exogenous sphingosine-1-phosphate (SPP) inhibits chemotactic motility of several transformed cell lines. We have found that SPP at high micromolar concentrations decreased chemotaxis of estrogen-independent (MDA-MB-231 and BT 549) and estrogen-dependent (MCF-7 and ZR-75-1) human breast cancer cells. Because SPP has been implicated as a lipid-signaling molecule with novel dual intra- and intercellular actions, it was of interest to determine whether the effect of SPP on chemotactic motility of human breast cancer cells is mediated intracellularly or through the recently identified endothelial differentiation gene (EDG) family of G protein-coupled SPP receptors. There was no detectable specific binding of [^{32}P]SPP to MDA-MB-231 or MCF-7 cells; however, reverse transcription-PCR analysis revealed that both MDA-MB-231 and MCF-7 cells expressed moderate levels of EDG-3, neither expressed EDG-1, and EDG-5 mRNA was expressed in MCF-7 but not in MDA-MB-231 cells. In contrast to SPP, sphinganine-1-phosphate, which binds to and signals through SPP receptors EDG-1, EDG-3, and EDG-5, had no effect on chemotactic motility of MDA-MB-231 or MCF-7 cells. To further discriminate between intracellular and receptor-mediated actions of SPP, we used caged SPP, a photolyzable derivative of SPP that elevates intracellular levels of SPP after illumination. Caged SPP inhibited chemotactic motility of MDA-MB-231 cells only upon UV irradiation. In addition, in MCF-7 cells, overexpression of sphingosine kinase, the enzyme that produces SPP, inhibited chemotactic motility compared with vector-transfected cells and markedly increased cellular SPP levels in the absence of detectable secretion. Our results suggest that the inhibitory effect of SPP on chemotactic motility of human breast cancer cells is likely mediated through intracellular actions of SPP rather than through cell surface receptors.

INTRODUCTION

The sphingolipid metabolite, SPP,[3] has been shown to act on various signaling pathways and to affect numerous biological functions (reviewed in Ref. 1). SPP has been implicated as a lipid second messenger in mitogenesis and calcium mobilization and also antagonizes ceramide-mediated apoptosis (1). The resting level of SPP is very low in most cells. The enzyme that catalyzes its formation, sphingosine kinase, is increased by a wide variety of stimuli, including mitogens, such as PDGF and fetal bovine serum (2–5), survival factors, such as nerve growth factor, basic fibroblast growth factor (6), and 12-*O*-tetradecanoylphorbol-13-acetate, (7), as well as 1α,25-dihydroxyvitamin D_3 (8), ligation of Fc receptors FcϵRI (9) and FcγRI (10), muscarinic acetylcholine receptors (11), and the B subunit of cholera toxin (12). The elevation in SPP as a result of sphingosine kinase activation is relatively short-lasting because of the action of two specific enzymes involved in its catabolism, SPP lyase and SPP phosphatase (13–17). Prevention of the increase in SPP by competitive inhibitors of sphingosine kinase selectively blocks cellular proliferation induced by PDGF and serum (2, 18), as well as FcϵRI- and FcγRI-mediated calcium release from internal sources (9, 10), calcium influx induced by carbachol (11), and the cytoprotective effects of 12-*O*-tetradecanoylphorbol-13-acetate, cyclic AMP activators (19, 20), nerve growth factor (6), and vitamin D_3 (8).

Although many studies indicate an intracellular site of action for SPP, pertussis toxin-sensitive G proteins have been shown to be involved in some of the signaling pathways regulated by SPP, suggesting that it might activate a receptor coupled to a G_i/G_o protein (21). In agreement, low concentrations of SPP activated G_i protein-gated inwardly rectifying potassium channels only when SPP was applied to the extracellular face of guinea pig atrial myocytes (22). Also, nanomolar concentrations of SPP (EC_{50}, 2 nM) rapidly induced Rho-dependent neurite retraction and cell rounding of mouse N1E-115 neurons (23) and platelet activation (24).

Recently, the GPCRs EDG-1, EDG-3, and EDG-5 were identified as high-affinity receptors for SPP (25–29). EDG-1 causes morphogenetic differentiation in response to SPP when expressed in HEK293 cells (25), whereas EDG-5, and to a lesser extent EDG-3, cause cell rounding in HEK293 cells and neurite retraction in PC12 cells (28). Binding of SPP to EDG-1, EDG-3, and EDG-5 stimulates different Gαs and $\beta\gamma$ dimers to signal through cyclic AMP, phospholipase C, Ras, mitogen-activated protein kinase, Rho, and several protein tyrosine kinases (25, 26, 29–32). Collectively, these studies suggest that SPP is capable of acting as a second messenger to regulate cell proliferation and survival and as a first messenger through the EDG family of GPCRs to regulate diverse biological responses.

Migration of cells is important in a variety of normal physiological processes, including embryogenesis, reproduction, inflammation, and wound healing. Moreover, cell motility plays a notable role in pathophysiological processes important for malignant progression such as metastasis. Cell migration is regulated by both expression of adhesion molecules and deposition of basement membrane or matrix proteins and soluble extracellular molecules interacting with specific cell surface receptors (33).

Previously, many studies have shown that exogenous SPP inhibits the chemotactic motility of various cancer cells at very low nanomolar concentrations (10–100 nM; Refs. 34–36). SPP also inhibits integrin-dependent motility (haptotactic motility) of mouse melanoma B16 cells by inhibiting actin nucleation and pseudopodia formation, without reducing integrin-dependent adhesion to the extracellular matrix (35). Furthermore, SPP immobilized on controlled pore glass beads inhibits motility of mouse melanoma cells (37), indicating that this effect may be mediated through cell surface receptors. In contrast, we found previously that inhibition of chemotactic motility of human breast cancer MCF-7 and MDA-MB-231 cells requires micromolar concentrations of SPP (38). Thus, it is of interest to determine whether SPP acts extracellularly as a ligand for cell surface receptors or

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[3] The abbreviations used are: SPP, sphingosine-1-phosphate; C8-Cer-1-P, *N*-octanoyl ceramide-1-phosphate; dihydro-SPP, sphinganine-1-phosphate; EDG, endothelial differentiation gene; FCM, fibroblast conditioned medium; GPCR, G protein-coupled receptors; IMEM, Richter's Improved Minimal Essential Medium; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription-PCR; FAK, focal adhesion kinase; GRAF, GTPase regulator associated with FAK; PMSF, phenylmethylsulfonyl fluoride.

intracellularly as a second messenger to inhibit chemotactic motility of human breast cancer cells.

The use of exogenously administered SPP is a major limitation of most previous studies because this approach cannot distinguish between receptor-mediated and intracellular effects because SPP is readily taken up by cells in culture (2, 26, 39). In this study, we demonstrated that motility of human breast cancer cells was inhibited when intracellular levels of SPP were increased either after photolysis of caged SPP or by overexpression of sphingosine kinase; both approaches bypass cell surface receptors. Our results suggest that SPP can inhibit motility through intracellular actions.

MATERIALS AND METHODS

Materials. SPP, dihydro-SPP, sphingosine, and *N,N*-dimethylsphingosine were purchased from Biomol Research Laboratory, Inc. (Plymouth Meeting, PA). SPP was >99% pure by TLC analysis. C8-SPP, C8-ceramide-1-phosphate, octyl β-D-glucopyranoside, and bovine brain ceramides (type IV) were from Calbiochem (La Jolla, CA). Cyclic SPP was purchased from Alexis Biochemicals (San Diego, CA). Other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). Medium, serum, and supplements were from Biofluids, Inc. (Rockville, MD); insulin and transferrin were from Collaborative Research (Lexington, MA); BSA, alkaline phosphatase (type VII-T), and Quik-fix kit were from Sigma Chemical Co. (St. Louis, MO); and G418 was from Mediatech (Herndon, VA). Collagen type IV was purchased from Collagen Corp. (Palo Alto, CA). Polycarbonate filters were from Poretics (Livermore, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

Cell Culture. The human breast cancer cell lines MDA-MB-231, MCF-7, BT 549, and ZR-75-1 were obtained from the Cell Culture Core Resource, Lombardi Cancer Center (Washington, DC). Cells were maintained in Richter's IMEM supplemented with 10% fetal bovine serum. Unless indicated, cells were seeded at 1.8×10^4 cells/cm^2. Twenty-four h prior to experiments, the medium was changed to serum-free IMEM without phenol red supplemented with 2 mM HEPES, 1% (v/v) nonessential amino acids, 1% (v/v) trace elements, 0.4% (v/v) insulin, transferrin, and selenium, 0.2% (w/v) fibronectin, and 1% (v/v) vitamins (38).

Chemotaxis Assay. Boyden chamber chemotactic motility assays were carried out essentially as described previously (38). Polycarbonate filters (13-mm diameter, 12-μm pore size) were coated with collagen IV (5 μg/filter) and then placed into the lower chamber. Collagen IV coating promotes uniform attachment to and migration across the filter, without formation of a barrier. FCM, obtained by incubating confluent NIH 3T3 cells for 48 h with IMEM supplemented with L-ascorbic acid (50 μg/ml), was placed in the lower chamber as chemoattractant. Cells were harvested by trypsinization and washed twice with IMEM containing 0.1% BSA, and 0.75×10^5 cells were added to the upper chamber and treated as indicated. The chambers were incubated in a humidified incubator at 37°C in 5% CO_2/95% air for 4 or 24 h as indicated. The cells that traversed the filter and spread on the lower surface of the filter were fixed and stained with Quik fix kit. Nonmigratory cells on the upper membrane surface were removed with a cotton swab. The number of migratory cells/membrane was determined using light microscopy with a ×10 objective. Each data point is the average number of cells in four random fields counted twice. Data are expressed as the means of counts from three individual wells ± SD.

SPP Binding Assay. [^{32}P]SPP was synthesized enzymatically using recombinant sphingosine kinase as described previously (26). The specific activity of [^{32}P]SPP was 6×10^6 cpm/pmol. Cells (10^5) were incubated with 1 nM [^{32}P]SPP in 200 μl of binding buffer [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 15 mM NaF, 2 mM deoxypyridoxine, 0.2 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin] for 30 min at 4°C. Cells were washed twice with 200 μl of ice-cold binding buffer containing 0.4 mg/ml fatty acid-free BSA and resuspended in PBS, and specific binding of [^{32}P]SPP was determined in the presence of 1 μM unlabeled SPP (26).

RT-PCR. Total RNA was isolated from cells using TRIZOL reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions and treated with RNase-free DNase I (RQ-1; Promega Corp., Madison, WI) to eliminate contaminating DNA. Reverse transcription was performed using MULV-RT (Perkin Elmer, Branchburg, NJ) for 15 min at 42°C. The primers (Life Technologies) used for PCR amplification were: 5′-GATATCATCGTCCGGCATTAC and 5′-ACCCTTCCCAGTGCATTGTTC for *EDG-1* (40); 5′-CACTCAGCAATGTACCTGTTCC and 5′-AACACCAGTACGATGGTGAC for *EDG-5* (41, 42); and 5′-GACTGCTCTACCATCTTGCCC and 5′-GTAGATGACAGGGTTCATGGC for *EDG-3* (43). PCR reactions were performed for 30 cycles with denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 50 s. The PCR products were analyzed by agarose gel electrophoresis after staining with ethidium bromide.

Expression of Sphingosine Kinase. SPHK1a was subcloned into a modified pcDNA3 vector (Invitrogen, Carlsbad, CA) to express it with an NH_2-terminal c-myc epitope tag by PCR using a 5′ primer with a *Bam*HI restriction site (5′-GAGGGATCCGAACCAGAATGCCCTCGAGGA-3′), and as the 3′ primer, the last 21 nucleotides of the SPHK1a sequence with an *Eco*RI overhang (5′-GAGGAATTCTTATGGTTCTTCTGGAGGTGG-3′). MCF-7 cells were seeded at 4×10^6 in 150-mm dishes and after 18 h, transfected with vector (c-myc-pcDNA3) or with the vector construct containing a sphingosine kinase insert (c-myc-pcDNA3-SPHK1a; Ref. 44) using Lipofectamine-Plus (Life Technologies, Inc.) according to the manufacturer's instructions. In some cases, cells were cotransfected with pCEFLGFP, which encodes the green fluorescent protein, and visualized with a fluorescence microscope as a measure of transfection efficiency. Stable transfectants were selected in the presence of 0.5 g/l G418 (44).

Western Blotting. Transfected cells were lysed in a buffer containing 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, 10 mM HEPES, 2 mM EDTA, 5 mM DTT, 1 mM PMSF, and 10 μg/ml of pepstatin A, leupeptin, and aprotinin. Lysates were centrifuged at $14,000 \times g$ for 15 min, and 20 μg of protein from the supernatants were separated by SDS-PAGE prior to immunoblotting with a monoclonal anti-c-myc (9E10) antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized with Super Signal chemiluminescent reagent (Pierce, Rockford, IL) using horseradish peroxidase conjugated antimouse IgG (1:5000).

Sphingosine Kinase Assay. Transfected cells were harvested and lysed by repeated freeze-thawing in buffer A [20 mM Tris (pH 7.4), 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine]. Sphingosine kinase activity was determined in the presence of 50 μM sphingosine, 0.25% Triton X-100, and [^{32}P]ATP (10 μCi, 20 mM) containing $MgCl_2$ (200 mM) in buffer A as described previously (45). The labeled SPP was separated by TLC on silica gel G60 with chloroform:methanol:acetone:acetic acid:water (10:4:3:2:1, v/v) and visualized by autoradiography. Radioactive spots corresponding to authentic SPP were identified as described (39) and quantified with a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA). Sphingosine kinase-specific activity was expressed as pmol of SPP formed per min per mg of protein.

Extraction of Lipids. Transfected cells were washed with PBS and scraped in 1 ml of 25 mM HCl/methanol. Lipids were extracted with 2 ml of chloroform:1 M NaCl (1:1, v/v) plus 100 μl of 3 N NaOH, and phases were separated (46). Phospholipid, ceramide, and sphingosine levels were determined in aliquots of the organic layer, whereas SPP levels were determined from aqueous phase extracts. Extraction of SPP from the medium of cells labeled with 40 μCi of [^{32}P$_i$] for 48 h was performed similarly. To 1 ml of medium, 2 ml of chloroform:methanol (1:1 v/v) plus 100 μl of 3 N NaOH were added, and phases were separated. The aqueous phase containing SPP was then acidified with 50 μl of concentrated HCl and reextracted twice with 1 ml of chloroform, and organic fractions were pooled (46).

Measurement of SPP Levels. SPP levels were measured essentially as described (46). Briefly, buffer B [200 mM Tris-HCl, (pH 7.4), 75 mM $MgCl_2$ in 2 M glycine (pH 9.0)] and 50 units of alkaline phosphatase were added to the aqueous phase containing extracted SPP. After incubating 1 h at 37°C, 50 μl of concentrated HCl were added, and sphingosine was extracted and quantitated with sphingosine kinase as described (46). For each experiment, known amounts of SPP were used to generate a standard curve.

Measurement of Sphingosine Levels. Sphingosine was measured by minor modifications of a method described previously (47). Briefly, aliquots of lipid extracts containing ≥50 nmol of total phospholipid were dried under nitrogen and resuspended in buffer A containing 0.25% Triton X-100, and

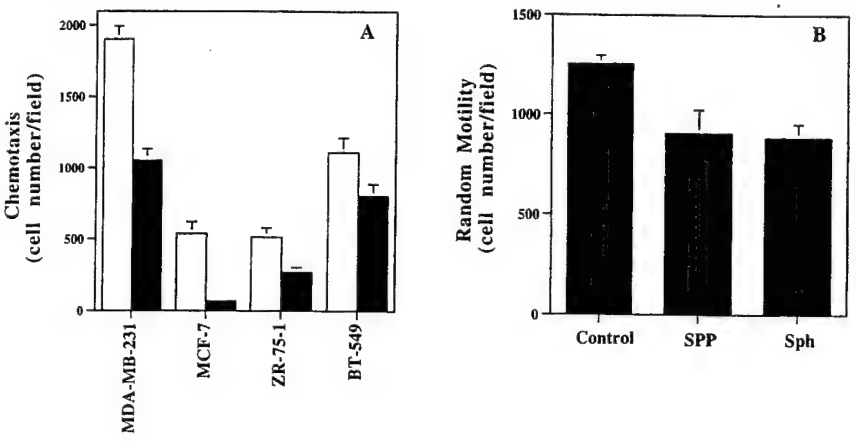

Fig. 1. SPP decreases motility of human breast cancer cells. *A*, effect of SPP on chemotaxis. MDA-MB-231, MCF-7, ZR-75-1, and BT-549 cells were allowed to migrate through polycarbonate filters coated with collagen IV for 4 h in the absence (□) or presence (■) of SPP (10 μM). FCM was present in the lower chamber as a chemoattractant, and chemotaxis was measured as described in "Materials and Methods." *B*, effect of SPP on random motility. MDA-MB-231 cells were incubated in the absence or presence of SPP (10 μM) or sphingosine (*Sph*, 15 μM), and random motility was measured in the absence of FCM. The results are from a representative experiment repeated three times. All treatments were significantly different from controls as determined by Student's *t* test ($P \leq 0.05$). *Bars*, SD.

sphingosine converted to SPP with sphingosine kinase was measured as described (47). For each experiment, known amounts of sphingosine were used to generate a standard curve.

Measurements of Ceramide Levels. Mass amounts of ceramide in cellular extracts were measured by the diacylglycerol kinase enzymatic method exactly as described (6).

Measurement of Total Cellular Phospholipids. Total phospholipids in cellular lipid extracts were quantified as described previously (48).

RESULTS

SPP Inhibits Chemotactic Motility of Human Breast Cancer Cells. In agreement with our previous report (38), we found that SPP inhibits chemotaxis of human breast cancer cells (Fig. 1*A*). SPP (10 μM) decreased chemotaxis of estrogen-independent MDA-MB-231 and BT 549 human breast cancer cells by 45 and 28%, respectively. Treatment of estrogen-dependent human breast cancer cells with SPP decreased chemotaxis by 87% (MCF-7) and 45% (ZR-75-1). In addition, treatment with high μM concentrations of SPP and sphingosine also slightly, but significantly, inhibited random motility of MDA-MB-231 cells (Fig. 1*B*).

Expression of SPP Receptors in MDA-MB-231 and MCF-7 Cells. It has been reported recently that SPP binds specifically and with high affinity to the GPCRs, EDG-1 (25), EDG-3, and EDG-5 (H218/AGR16; Ref. 28). We therefore examined expression of SPP receptor mRNAs in MCF-7 and MDA-MB-231 cells using RT-PCR analysis. The entire open reading frame of each of the three SPP receptors is encoded within a single exon (49, 50), and it is not possible to design PCR primers that span an intron junction. Therefore, controls without reverse transcriptase were performed in all cases to ensure that the product observed was not derived from genomic DNA (Fig. 2). Expression of *EDG-3* mRNA was detected in MDA-MB-231 cells; however, neither *EDG-1* nor *EDG-5* mRNA could be detected. MCF-7 cells expressed *EDG-3* as well as *EDG-5*, but similarly to MDA-MB-231 cells, did not express *EDG-1*. MCF-7 cells stably overexpressing *EDG-1* were included as a positive control. Digestion of the human RT-PCR products with restriction enzymes gave fragments of expected sizes, confirming their identity (data not shown).

To confirm that SPP receptors were constitutively expressed in MCF-7 and MDA-MB-231 cells, we attempted to measure specific binding of [^{32}P]SPP. In both cell lines, total binding of 1 nM [^{32}P]SPP was low and was not significantly reduced by the addition of 1000-fold excess of unlabeled SPP, indicating very low expression of specific SPP receptors by these cells. The failure to detect specific SPP binding is not a result of improper binding conditions, because in MCF-7 cells overexpressing EDG-1, significant specific SPP binding could be detected (10.3 ± 0.6 fmol/10^5 cells).

Effects of SPP Analogues on Chemotaxis. Low nanomolar concentrations of SPP have been shown to inhibit chemotaxis of human melanoma cells (34, 37). In contrast, although high concentrations of SPP markedly inhibit chemotaxis of MDA-MB-231 cells, low concentrations of SPP (from 10 nM to 1 μM) had no significant effects (Fig. 3*A*). Previously, we found that similar to SPP, high concentrations of sphingosine also inhibit chemotaxis of these cells (38). Thus, it was of interest to compare the effects of several other structurally related SPP analogues (Fig. 3*A*). None of the other SPP analogs tested, including the short-chain SPP analogue C8-SPP, C8-Cer-1-P, cyclic SPP, or caged SPP (see below), significantly inhibited motility of MDA-MB-231 cells. Because of the structural similarities between LPA and SPP and because they have been reported to bind to the homologous receptors EDG-2 and EDG-4 (29), we also examined the effect of LPA. Recently, it has been suggested that LPA is a low-affinity ligand for EDG-1 (51). However, LPA, even at concentrations as high as 10 μM, had no significant effect on chemotaxis of MDA-MB-231 cells (Fig. 3*A*).

EDG-1, EDG-3, and EDG-5, bind SPP with high specificity. Di-

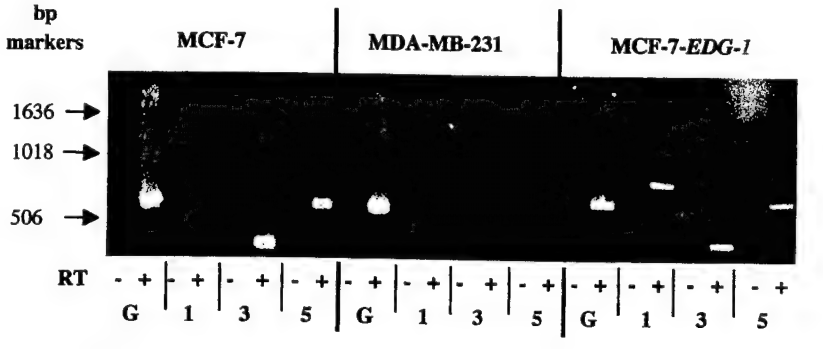

Fig. 2. Detection of EDG receptor mRNA. RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (*G*), *EDG-1* (*1*), *EDG-3* (*3*), and *EDG-5* (*5*) mRNA expression was performed on RNA isolated from MCF-7 MDA-MB-231 and MCF-7 cells stably expressing myc epitope-tagged *EDG-1* (*MCF-7-EDG-1*), without (−) or with (+) MULV-RT (*RT*) as described in "Materials and Methods." Similar results were obtained in two independent experiments.

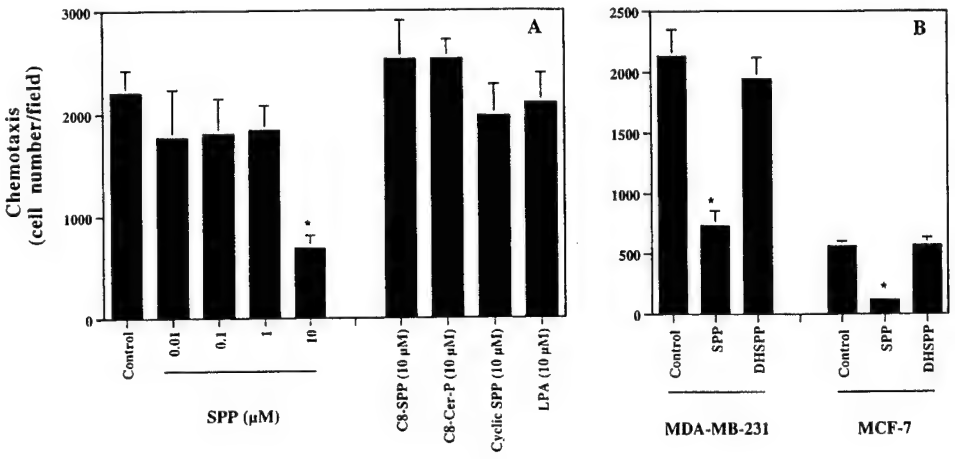

Fig. 3. Effect of structurally related SPP analogues on chemotaxis. *A*, MDA-MB-231 cells were treated without or with the indicated concentrations of SPP, 10 μM C8-SPP, C8-Cer-1-P, cyclic-SPP, or LPA, and chemotaxis was determined. *B*, differential effects of dihydro-SPP and SPP on motility. MDA-MB-231 or MCF-7 cells were treated without or with 10 μM SPP or dihydro-SPP (*DHSPP*), and chemotactic motility was determined. The results are from a representative experiment repeated at least two times. *, significant differences from untreated controls as determined by Student's *t* test ($P \leq 0.05$). *Bars*, SD.

hydro-SPP, which is similar to SPP but lacks the *trans* double bond, binds to and signals through all three SPP receptors with similar potency to SPP (25, 26, 28). Thus, if inhibition of chemotactic motility is mediated through one or more of these SPP receptors, dihyro-SPP should mimic the effect of SPP. However, 10 μM dihydro-SPP had no significant effect on chemotactic motility of MDA-MB-231 or MCF-7 cells (Fig. 3*B*). Collectively, these data suggest that the ability of SPP to inhibit chemotaxis may not be related to binding to EDG cell surface receptors.

Inhibition of Chemotaxis by Caged SPP. To determine whether SPP formed intracellularly regulates cell motility, it is useful to be able to experimentally increase intracellular SPP bypassing cell surface receptors. Recently, we synthesized a photolyzable derivative of SPP, termed caged SPP, which is taken up by cells and upon UV irradiation, is photolyzed to form SPP and *o*-nitrosoacetophenone (52). The introduction of caged SPP into cultured cells permits rapid and controlled elevation of intracellular SPP levels upon illumination (data not shown). The effect of caged SPP photolysis on chemotactic motility of MDA-MB-231 cells was compared with that of exogenously added SPP (Fig. 4). Exogenous SPP (10 μM) inhibited chemotactic motility of MDA-MB-231 cells by 50%, whereas caged SPP (5 or 10 μM) had no significant effect on chemotactic motility in nonilluminated cells or in cells illuminated prior to its addition. However, UV irradiation of caged SPP-loaded cells caused strong inhibition of chemotaxis, whereas UV irradiation alone had no effect (Fig. 4), nor did it alter the inhibitory effect of exogenous SPP. To exclude the possibility that the by product of caged SPP photolysis, *o*-nitrosoacetophenone, may affect chemotaxis, the motility of cells treated with caged cyclic SPP was measured. Unlike caged SPP, caged cyclic SPP had no effect on chemotaxis of MDA-MB-231 cells with or without UV irradiation (Fig. 4). Moreover, incubation of cells with *o*-nitrosoacetophenone had no effect on chemotaxis, even when cells were incubated for up to 4 h at very high concentrations (100 μM; data not shown).

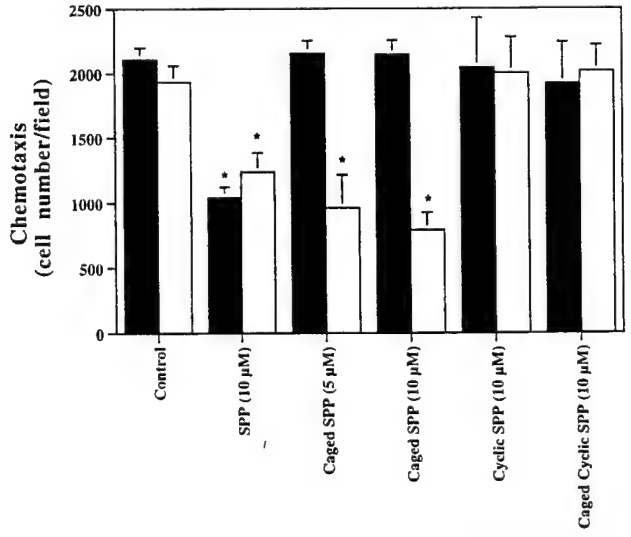

Fig. 4. Effect of caged SPP on chemotactic motility. MDA-MB-231 cells were incubated without (control) or with the indicated concentrations of caged SPP, SPP, cyclic SPP, or caged cyclic SPP for 2 h. Cells were then collected and washed twice with IMEM containing 4 mg/ml fatty acid-free BSA, and chemotaxis was measured without (■) or with (□) UV irradiation for 30 s. Data are means of triplicate determinations; *bars*, SD. The results are from a representative experiment repeated three times. *, significant differences from controls as determined by Student's *t* test ($P \leq 0.05$).

Overexpression of Sphingosine Kinase Decreases Chemotactic Motility of MCF-7 Cells. As an alternative method to increase the level of intracellular SPP, MCF-7 cells were transfected with a murine sphingosine kinase expression vector (44). Transient expression of sphingosine kinase in MCF-7 cells decreased chemotaxis toward FCM (Fig. 5*A*). Moreover, although the number of migrating cells was much lower in the absence of chemoattractant, transient expression of sphingosine kinase also significantly reduced this random motility (Fig. 5*A*).

To further examine the effect of overexpression of sphingosine kinase on chemotaxis and to correlate this with protein expression, MCF-7 cells were transfected with c-myc-tagged sphingosine kinase and selected on the basis of G418 resistance. Pools of stably transfected cells were used to avoid potential phenotypic changes attributable to selection and propagation of clones derived from single individual cells. Stable expression of sphingosine kinase decreased chemotactic motility of MCF-7 cells by 30 and 46% after 4 and 24 h, respectively (Fig. 5*B*). Sphingosine kinase activity in the cytosolic fraction of these transfected cells was elevated by 36-fold over control vector-transfected cells (Table 1), whereas SPP levels were elevated by only 3.3-fold (Table 1). This is in agreement with previous results (44), where the large fold increase in transfected sphingosine kinase activity measured *in vitro* did not correspond with the fold increase in SPP levels. To further examine the correlation between expression of sphingosine kinase and increased SPP levels with inhibition of chemotaxis, we generated a separate independent pool of transfected cells that express even higher levels of sphingosine kinase (Table 1). Western blot analysis of the cytosolic fractions of these cells using anti-c-myc antibody revealed a band migrating at the expected molecular weight of c-myc-tagged sphingosine kinase, which was absent in vector-transfected cells (Fig. 6*A*, *inset*). Interestingly, although

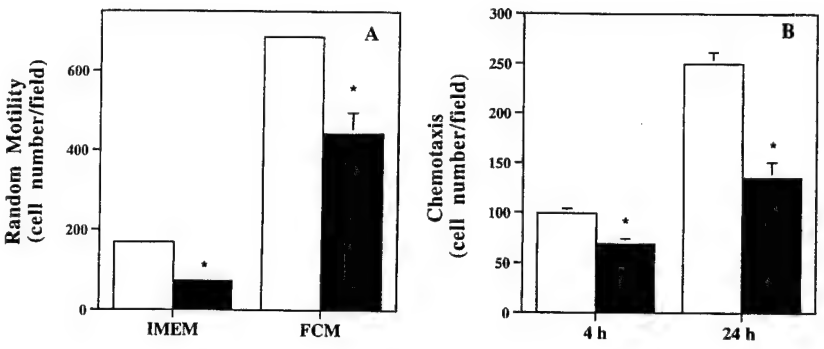

Fig. 5. Overexpression of sphingosine kinase decreases chemotactic motility of MCF-7 cells. *A*, MCF-7 cells transiently cotransfected with pCEFLGFP and either vector c-myc-pcDNA3 (□) or c-myc-pcDNA3-SPHK1a (■) were trypsinized after 48 h, washed with serum-free medium, and allowed to migrate in a Boyden chamber for 4 h toward either medium alone (*IMEM*) as a measure of random motility or toward *FCM* as chemoattractant to measure chemotaxis. *Bars*, SD. *B*, pooled clones of MCF-7 cells stably expressing c-myc-pcDNA3 (□) or c-myc-pcDNA3-SPHK1a (■) were allowed to migrate toward FCM for 4 or 24 h, and chemotaxis was measured. *, significant differences from untreated controls as determined by Student's *t* test ($P \leq 0.05$). *Bars*, SD.

expression of sphingosine kinase in this second pool of transfected MCF-7 cells resulted in even more marked increase in mass levels of SPP (Fig. 6*B*), there were no concomitant changes in the other sphingolipid metabolites, sphingosine (Fig. 6*C*), or ceramide (Fig. 6*D*). Nevertheless, chemotaxis of the MCF-7 cells with higher sphingosine kinase expression and cellular SPP levels was more strongly inhibited (Fig. 6*E*).

Because it has been suggested that SPP inhibits melanoma cell motility through an extracellular action by specific binding to cell surface receptors (37), it was of interest to determine whether MCF-7 cells overexpressing sphingosine kinase, which have increased cellular levels of SPP, can secrete SPP. We have recently developed a sensitive assay for the measurement of SPP (46) that is able to detect as little as 1 pmol of SPP. Using this assay, we were unable to detect secretion of SPP into the medium by either of the stable pools of sphingosine kinase-transfected MCF-7 cells (Table 1), suggesting that SPP is not released by these cells in appreciable amounts. To increase the sensitivity of detection of secreted SPP, we also labeled cells to isotopic equilibrium with [^{32}P]P_i and analyzed the labeled SPP in cells as well as in the medium. Despite the large increases in [^{32}P]SPP detected in MCF-7 cells overexpressing sphingosine kinase, there was no detectable labeled SPP released into the medium. On the basis of the sensitivity of these methods, we estimate that the concentration of SPP in the extracellular medium is ≤0.4 nM, a concentration well below the K_d for binding of SPP to its EDG receptors (26, 28).

DISCUSSION

SPP has been shown to function both intracellularly as a second messenger and at the cell surface through specific SPP receptors to regulate numerous biological processes (29, 53). Previously, based on addition of SPP to cells, many studies have suggested that SPP inhibits cell motility and chemotaxis by binding to a putative cell surface receptor (reviewed in Ref. 37). Recently, EDG-1 was identified as a high-affinity (K_d, 8 nM), specific receptor for SPP (25). Two related receptors, EDG-3 and EDG-5 (also known as AGR16/H218), also bind SPP with low nanomolar affinities (28). However, it has not yet been determined whether EDG-1, EDG-3, or EDG-5 plays a role in mediating the effects of exogenous SPP on inhibition of motility.

SPP has been shown to inhibit cell motility, chemoinvasion, and haptotactic motility (35) of human B16 melanoma cells in a low concentration range (10–100 nM; Ref. 34). These effects appear to be mediated through a cell surface receptor, because SPP immobilized on glass beads, which cannot traverse the cell membrane, mimicked the effects of SPP (37). SPP also inhibited the chemotactic motility and transendothelial migration of human neutrophils (36) and PDGF-induced chemotaxis of aortic smooth muscle cells (3) at nanomolar concentrations. In contrast, micromolar concentrations of SPP were necessary to inhibit chemotactic motility of human breast cancer cell lines (MCF-7 and MDA-MB-231; Ref. 38) and human HT1080 fibrosarcoma cells (34). It was thus important to determine whether inhibition of breast cancer cell motility by SPP is mediated intracellularly or through a cell surface receptor.

Surprisingly, in this study, several lines of evidence indicated that SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells through an intracellular action rather than by signaling through cell surface receptors: (*a*) SPP had no effect on motility of MDA-MB-231 cells at concentrations below 1 μM, approximately two orders of magnitude higher than the K_d for binding of SPP to EDG-1, EDG-3, or EDG-5 (26, 28); (*b*), although MDA-MB-231 cells express SPP receptor EDG-3 mRNA and MCF-7 cells express EDG-3 and EDG-5, no specific SPP binding could be detected to either MCF-7 or MDA-MB-231 cells, indicating that either the receptor proteins are not present on the cell surface or that they are expressed at very low levels; and (*c*), dihydro-SPP, which binds to and signals through all three SPP receptors (26, 28)[4] had no effect on the chemotactic motility of MDA-MB-231 or MCF-7 cells. In agreement, it was reported previously that dihydro-SPP did not affect chemotactic motility of human neutrophils (36). Moreover, sphingosine, which is rapidly taken up by cells and converted intracellularly to SPP by sphingosine kinase, also inhibits chemotaxis of MCF-7 and MDA-MB-231 cells (38).

Additionally, two independent approaches were used to elevate intracellular SPP, bypassing cell surface SPP receptors: treatment of cells with caged SPP, which is taken up by cells and forms SPP intracellularly upon UV irradiation (52); and overexpression of sphingosine kinase, the enzyme that forms SPP within cells. After UV photolysis of intracellular caged SPP, chemotactic motility was inhibited to the same extent as after treatment with exogenous SPP. Overexpression of sphingosine kinase by transfection in MCF-7 cells led to increased intracellular SPP and drastically inhibited chemotactic motility as well as random motility. Although the intracellular levels of SPP were elevated 3–4.6-fold in stably transfected cells, no

Table 1 *Sphingosine kinase activity and SPP levels in pooled clones of MCF-7 cells stably expressing sphingosine kinase*

Pooled clones of MCF-7 cells stably transfected with c-myc-pcDNA3-SPHK1a or vector alone (c-myc-pCDNA3) were washed and incubated in serum-free medium overnight. Cytosolic fractions were prepared, and sphingosine kinase activity was measured as described in "Materials and Methods." Data are expressed as fold increases compared with respective controls and are means ± SD of triplicate determinations from a representative experiment. Cellular and secreted SPP levels were measured after 24 h in serum-free medium.

	Pool 1	Pool 2
Sphingosine kinase activity (fold increase)	36 ± 3	115 ± 7
Cellular SPP levels (fold increase)	3.3 ± 0.4	4.6 ± 0.2
Secreted SPP (pmol/ml)	ND[a]	ND

[a] ND, not detected (≤0.4 pmol/ml).

[4] J. R. Van Brocklyn and S. Spiegel, unpublished observations

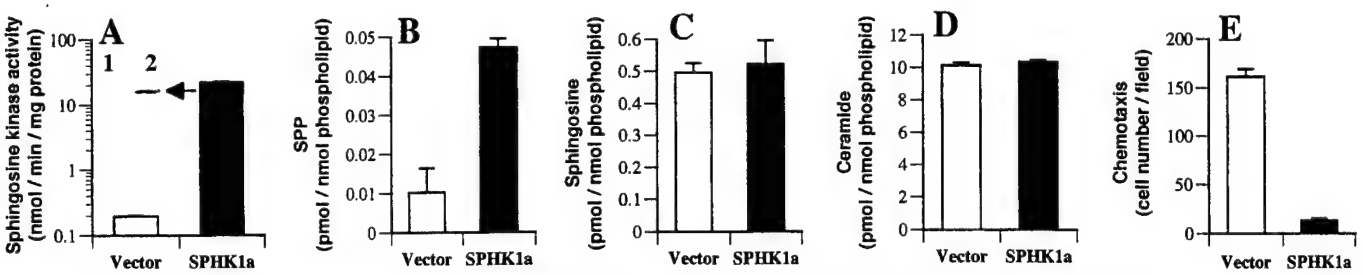

Fig. 6. Changes in mass levels of sphingolipid metabolites in pooled clones of MCF-7 cells overexpressing sphingosine kinase and corresponding inhibition of motility. Pooled clones of MCF-7 cells expressing c-myc-pCDNA3-SPHK1a (■) or vector alone (c-myc-pCDNA3; □) were washed and incubated in serum-free medium overnight. Sphingosine kinase activity (*A*), mass levels of SPP (*B*), sphingosine (*C*), and ceramide (*D*) were measured as described in "Materials and Methods." Data are means of triplicate determinations from a representative experiment; *bars*, SD. *A, (inset)*, Western blot of MCF-7 cell lines expressing c-myc-pcDNA3 (*Lane 1*) or c-myc-SPHK1a (*Lane 2*), respectively. Equal amounts of protein (20 μg) were analyzed by immunoblotting after SDS-PAGE. *Arrowhead*, migration of myc-tagged SPHK1a. *E*, these transfected cells were allowed to migrate toward either medium alone (IMEM) or FCM for 4 h, and chemotaxis was measured. Data are means of triplicate determinations; *bars*, SD.

detectable amounts of SPP were released into the medium. Thus, it seems highly unlikely that SPP inhibits motility of breast cancer cells by binding to cell surface receptors.

Although we have now shown that elevation of the intracellular levels of SPP inhibits motility of human breast cancer cells, our studies cannot exclude the possibility that in other cell types, the mode of action of SPP may vary, depending on the expression of different SPP receptors and the signaling pathways that the receptors couple to in different cell types. It is possible that SPP could effect the same biological response through separate mechanisms, even in the same cells. For example, microinjected SPP is mitogenic for Swiss 3T3 cells, however, to a lesser degree than exogenously added SPP (26). Although the mitogenic effect of microinjected SPP is insensitive to pertussis toxin treatment, the effect of exogenous SPP is partially inhibited by pertussis toxin, decreasing the response to approximately the same level as that induced by microinjection (26). These results suggested that in Swiss 3T3 cells, which express all three known SPP receptors, the mitogenic effect of SPP is attributable to the additive contributions of receptor-mediated responses and intracellular actions. Thus, it is possible that cell motility could also be regulated by both intracellular actions of SPP, as in breast cancer cells, and possibly by receptor-mediated actions of SPP, as in melanoma cells.

The mechanism of the inhibitory effect of SPP on motility has not yet been elucidated. It is possible that the inhibitory effect of SPP on chemotactic motility may be mediated by FAK and Rho. Activation of Rho results in the formation of actin stress fibers and focal adhesions (54–56), and it is well established that cytoskeletal changes regulated by Rho GTPases form the basis for the coordination of cell motility (57, 58). SPP induces tyrosine phosphorylation of FAK and actin stress fiber formation in a Rho-dependent manner (59). Although the exact connection between the expression and tyrosine phosphorylation of FAK and cell motility is not completely understood, it has been suggested that maximal migration requires optimal levels of FAK tyrosine phosphorylation (60), and inhibition of FAK signaling in focal adhesions decreases cell motility (61). Intriguingly, it was found recently that GRAF, which preferentially stimulates the GTPase activity of RhoA and Cdc42, can regulate cytoskeletal changes induced by SPP (62). In Swiss 3T3 cells, where GRAF is not expressed at detectable levels, GRAF overexpression inhibited SPP-induced, Rho-mediated stress fiber formation. Conversely, in PC12 cells that express high levels of GRAF, overexpression of GRAF stimulated Rho-mediated neurite retraction induced by SPP (62). These results may suggest that an optimal level of Rho activity is required for maximal cell motility. Another possibility is that the effect of SPP on motility is related to regulation of cytosolic free calcium. It has been shown that large (micromolar) global increases in the cytosolic free calcium concentration in neutrophils are usually associated with termination of chemotaxis (63). Recently, it was found that cells move forward during the phase of transient calcium elevation and remain stationary during the troughs (64). Consequently, changes in the frequency of Ca^{2+} fluctuations directly affect cerebellar granule cell movement, *i.e.*, reducing the frequency of calcium fluctuations slows down the speed of cell movement (64). Interestingly, SPP has been found to elicit a nonoscillatory increase of intracellular free calcium in CG4, NIH 3T3, and PDGF receptor-transfected PC12 cells (65). Moreover, inhibition of sphingosine kinase with DL-*threo*-dihydrosphingosine significantly reduced the percentage of cells responding to PDGF exposure with calcium oscillations in transformed oligodendrocytes (66). Thus, it is possible that SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells by inducing a nonoscillatory increase in intracellular free calcium. Additional studies are needed to clarify the importance of different signaling pathways for controlling motility that are regulated by SPP. Because cell movement is considered an important step in invasion and metastasis of cancer cells, the finding that endogenous SPP regulates cell migration and chemotactic signaling may have substantial biological ramifications.

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Sphingosine Kinase Expression Increases Intracellular Sphingosine-1-phosphate and Promotes Cell Growth and Survival

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Abstract. Sphingosine-1-phosphate (SPP) is a bioactive lipid that has recently been identified as the ligand for the EDG family of G protein–coupled cell surface receptors. However, the mitogenic and survival effects of exogenous SPP may not correlate with binding to cell-surface receptors (Van Brocklyn, J.R., M.J. Lee, R. Menzeleev, A. Olivera, L. Edsall, O. Cuvillier, D.M. Thomas, P.J.P. Coopman, S. Thangada, T. Hla, and S. Spiegel. 1998. *J. Cell Biol.* 142:229–240). The recent cloning of sphingosine kinase, a unique lipid kinase responsible for the formation of SPP, has provided a new tool to investigate the role of intracellular SPP. Expression of sphingosine kinase markedly increased SPP levels in NIH 3T3 fibroblasts and HEK293 cells, but no detectable secretion of SPP into the medium was observed. The increased sphingosine kinase activity in NIH 3T3 fibroblasts was sufficient to promote growth in low-serum media, expedite the G_1/S transition, and increase DNA synthesis and the proportion of cells in the S phase of the cell cycle with a concomitant increase in cell numbers. Transient or stable overexpression of sphingosine kinase in NIH 3T3 fibroblasts or HEK293 cells protected against apoptosis induced by serum deprivation or ceramide elevation. *N,N*-Dimethylsphingosine, a competitive inhibitor of sphingosine kinase, blocked the effects of sphingosine kinase overexpression on cell proliferation and suppression of apoptosis. In contrast, pertussis toxin did not abrogate these biological responses. In Jurkat T cells, overexpression of sphingosine kinase also suppressed serum deprivation- and ceramide-induced apoptosis and, to a lesser extent, Fas-induced apoptosis, which correlated with inhibition of DEVDase activity, as well as inhibition of the executionary caspase-3. Taken together with ample evidence showing that growth and survival factors activate sphingosine kinase, our results indicate that SPP functions as a second messenger important for growth and survival of cells. Hence, SPP belongs to a novel class of lipid mediators that can function inside and outside cells.

Key words: sphingosine kinase • sphingosine-1-phosphate • cell growth • apoptosis

SPHINGOSINE-1-phosphate (SPP),[1] a phosphorylated derivative of sphingosine, the structural backbone of all sphingolipids, is a bioactive lipid that regulates diverse biological processes, such as calcium mobilization, cell growth, differentiation, survival, motility, and cytoskeleton organization (Goetzl and An, 1998; Spiegel, 1999). Recent interest in SPP has been stimulated by the discovery of a family of cell surface G-protein–coupled receptors (GPCR), encoded by the endothelial differentiation genes (EDG), that specifically bind SPP with high affinity and specificity (An et al., 1997; Goetzl and An, 1998; Lee et al., 1998; Van Brocklyn et al., 1998, 1999; Kon et al., 1999), supporting a role for SPP as an extracellular mediator. Indeed, previously, some of the biological effects of SPP when added exogenously, such as inhibition of platelet activation and motility and cell shape changes (Yamamura et al., 1997), induction of neurite retraction and soma rounding (Postma et al., 1996; Sato et al., 1997), and activation of G_i protein–gated inward rectifying K^+ channels in atrial myocytes (van Koppen et al., 1996), have been attributed to interactions with putative cell surface receptors. SPP receptors, EDG-1, -3, and -5, couple to different $G\alpha s$ and $\beta\gamma$ dimers to signal through cAMP, phospholipase C, Ras, mitogen-activated protein kinase, Rho, and

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1. *Abbreviations used in this paper:* Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; BrdU, bromodeoxyuridine; DMS, *N,N*-dimethylsphingosine; EDG, endothelial differentiation gene; GFP, green fluorescent protein; GPCR, G protein coupled receptors; SPHK, sphingosine kinase; SPP, sphingosine-1-phosphate; TNF, tumor necrosis factor.

several protein tyrosine kinases (An et al., 1997; Goetzl and An, 1998; Lee et al., 1998; Okamoto et al., 1998; Van Brocklyn et al., 1998; Zondag et al., 1998; Gonda et al., 1999). Furthermore, SPP is stored in high concentrations in human platelets, from which it is released upon activation by physiological stimuli (Yatomi et al., 1997a), suggesting that SPP can be considered an autocrine factor involved in endothelial injury, inflammation, thrombosis, and angiogenesis.

However, this sphingolipid metabolite also has many of the hallmarks of classical second messengers (reviewed in Spiegel et al., 1996). The level of SPP is very low in cells and is rapidly increased by activation of sphingosine kinase induced by diverse physiological stimuli, including PDGF (Olivera and Spiegel, 1993; Bornfeldt et al., 1995; Coroneos et al., 1995; Pyne et al., 1996), NGF (Edsall et al., 1997; Rius et al., 1997), muscarinic acetylcholine agonists (Meyer zu Heringdorf et al., 1998), tumor necrosis factor (TNF)-α (Xia et al., 1998), activation of PKC (Mazurek et al., 1994; Buehrer et al., 1996), and cross-linking of the immunoglobulin receptors FcεR1 (Choi et al., 1996) and FcγR1 (Melendez et al., 1998). Similar to other signaling molecules, SPP has a short half-life due to rapid turnover catalyzed by SPP lyase and/or SPP phosphatase (Van Veldhoven and Mannaerts, 1991, 1994; Saba et al., 1997; Mandala et al., 1998; Zhou and Saba, 1998). Moreover, prevention of SPP formation by competitive inhibitors of sphingosine kinase blunted the mitogenic response to PDGF (Olivera and Spiegel, 1993; Rani et al., 1997), the cytoprotective effects of NGF (Edsall et al., 1997), vitamin D3 (Okazaki et al., 1998), PKC, and cAMP activators (Cuvillier et al., 1996; Machwate et al., 1998), as well as calcium mobilization induced by FcεR1, FcγR1, and muscarinic acetylcholine receptors (Choi et al., 1996; Melendez et al., 1998; Meyer zu Heringdorf et al., 1998). In addition, microinjected SPP mobilizes calcium from internal sources (Meyer zu Heringdorf et al., 1998), is mitogenic for Swiss 3T3 fibroblasts (Van Brocklyn et al., 1998), and inhibits apoptosis of mouse oocytes induced by the antitumor drug doxorubicin (Perez et al., 1997). Collectively, these observations provide new insights into the biological functions of SPP and emphasize the importance of sphingosine kinase, the enzyme responsible for SPP formation, whether it functions inter- or intracellularly. Until recently, it had been difficult to critically evaluate the second messenger roles of SPP because most of the relevant enzymes involved in its metabolism had not been purified and cloned. Thus, much of the evidence that led to the elucidation of its importance has been indirect, relying on exogenous application and the use of inhibitors, and is further complicated by the existence of cell-surface receptors. We recently purified sphingosine kinase to apparent homogeneity from rat kidney (Olivera et al., 1998) and subsequently cloned and characterized the first mammalian sphingosine kinases (murine SPHK1a and SPHK1b) (Kohama et al., 1998). In this study, we demonstrate that overexpression of sphingosine kinase markedly increased intracellular mass levels of SPP, enhanced proliferation by promoting the G_1 to S phase transition, and suppressed serum deprivation- and ceramide-induced apoptosis. Because these effects were mediated in the absence of detectable secretion of SPP and were pertussis toxin independent, our results support an additional role for SPP as an intracellular second messenger important for cell proliferation and survival.

Materials and Methods

Materials

SPP, dihydroSPP, sphingosine, *N,N*-dimethylsphingosine, and C2-ceramide were from Avanti Polar Lipids Research Laboratory Inc. [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham Corp. Alkaline phosphatase (bovine intestinal mucosa, type VII-NT) and pertussis toxin were from Sigma Chemical Co. Select-Amine medium, and G418 were obtained from Biofluids, Inc. Restriction enzymes were from New England Biolabs Inc. Monoclonal antibodies against c-myc were from Zymed, and anti–mouse Texas red dye–conjugated goat antibody was from Jackson ImmunoResearch Laboratories, Inc. The Anti-Fade kit was from Molecular Probes. The bromodeoxyuridine incorporation detection kit and anti–mouse FITC-conjugated IgG were obtained from Boehringer Mannheim Biochemicals. Bisbenzimide hydrochloride (Hoechst 33258) was from Calbiochem Corp. Acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) was from Bachem. Anti-Fas IgM (clone CH-11) was from Upstate Biotechnology Inc.

Cell Culture

NIH 3T3 fibroblasts (ATCC CRL-1658) and human embryonic kidney cells (HEK293, ATCC CRL-1573) were grown in high glucose DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine supplemented with 10% calf serum and fetal bovine serum, respectively (Kohama et al., 1998). Jurkat T leukemia cells were cultured in RPMI 1640 containing 10% fetal bovine serum. Before addition of exogenous stimuli, cells were resuspended at a density of 0.75–1 × 10^6 cells/ml in medium containing 5 μg/ml transferrin and 5 μg/ml insulin in place of serum (Cuvillier et al., 1998).

Cloning and Expression of Sphingosine Kinase

SPHK1a (accession number AF068748) was subcloned into a modified pcDNA3 vector (Invitrogen Corp.) to express proteins with an NH_2-terminal c-myc epitope tag (a gift from Dr. Peter Burbelo, Georgetown University, Washington, DC) by PCR using a 5′ primer with a BamH1 restriction site (5′-GAGGGATCCGAACCAGAATGCCCTCGAGGA-3′), and as the 3′ primer, the last 21 nucleotides of the SPHK1a sequence with an EcoRI overhang (5′-GAGGAATTCTTATGGTTCTTCTG-GAGGTGG-3′). For transient expression, plasmids were transfected into cells using Lipofectamine Plus (Life Technologies, Inc.) according to the manufacturer's instructions at a 5:1 ratio with pCEFL-GFP, which encodes green fluorescent protein (GFP; a generous gift of Dr. Silvio Gutkind, NIH, Bethesda, MD). Transfection efficiencies were typically 30 and 40% for NIH 3T3 and HEK293 cells, respectively. Jurkat T cells were transfected by electroporation at a cell density of 10^6 cells/ml with 15 μg of DNA using a Gene Pulser apparatus (Bio-Rad Laboratories) at 400 V and 960 μFa. Stable transfectants containing pcDNA3 plasmids were selected in medium containing 1 mM sodium pyruvate and 0.5 g/liter G418 (NIH 3T3 fibroblasts) or 1 g/liter G418 (HEK293 and Jurkat cells). For all experiments, nonclonal pools of stably transfected cells were used to avoid clonal variability.

Measurement of SPP

Cells were incubated in low serum or serum-free media for at least 24 h. The media was then removed, cells were washed with PBS and scraped in 1 ml 25 mM HCl/methanol, and SPP levels were measured essentially as described (Edsall and Spiegel, 1999). In brief, lipids from the cells were then extracted with 5 ml of chloroform/methanol/1 M NaCl (2:1:2, vol/vol) containing 100 μl 3 M NaOH. SPP is water soluble at alkaline pH, and partitions into the aqueous phase. SPP in the aqueous phase was dephosphorylated with alkaline phosphatase (25–50 U) in buffer containing 1.2 M glycine buffer pH 9.0 and 75 mM $MgCl_2$. After 1 h at 37°C, 40 μl concentration. HCl was added, and sphingosine was extracted and quantitated by phosphorylation with sphingosine kinase and [^{32}P]ATP (Olivera and Spiegel, 1998). Total phospholipids in cellular lipid extracts were quantified by a colorimetric reaction with malachite green exactly as previously described (Edsall et al., 1997).

Measurement of [^{32}P]SPP Release

HEK293 cells were grown to subconfluency in 100-mm dishes in 10% FBS media, washed and incubated in serum-free media containing 40 μCi/ml [^{32}P]orthophosphate for 24 h to label the phospholipid pools to isotopic equilibrium. In some experiments, sphingosine (5 μM) was added to the media 10 min before termination of the 24-h incubation period. The media was then collected and the cells scraped from the dishes. Cellular and secreted [^{32}P]SPP were extracted in alkaline conditions as described above, followed by acidic extraction with chloroform/methanol/concentrated HCl (100:100:1, vol/vol), to partition [^{32}P]SPP into the organic phase. [^{32}P]SPP was resolved on TLC with 1-butanol/ethanol/acetic acid/water (80:20:10:20, vol/vol), visualized and quantified as described (Olivera et al., 1998).

Assay of Sphingosine Kinase Activity

Cells were incubated in low serum or serum-free media for at least 24 h, and then harvested and lysed by freeze-thawing in buffer containing 20 mM Tris, pH 7.4, 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β-glycero-phosphate, 15 mM NaF, 10 μg/ml leupeptin, aprotinin and soybean trypsin inhibitor, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine. Cell lysates were fractionated into cytosol and membrane fractions by centrifugation at 100,000 *g* for 60 min 4°C. Sphingosine kinase activity was determined in the presence of 50 μM sphingosine, dissolved in 5% Triton X-100 (final concentration 0.25%), and [^{32}P]ATP (10 μCi, 1 mM) containing $MgCl_2$ (10 mM) as previously described (Kohama et al., 1998), and specific activity was expressed as picomoles of SPP formed per minute per milligram protein.

Immunostaining

Cells grown on glass coverslips coated with collagen I were incubated overnight in DMEM supplemented with 2 μg/ml insulin, 2 μg/ml transferrin, and 20 μg/ml BSA. Cells were washed with PBS and fixed in 3.7% formalin and 0.1% Triton X-100 for 20 min. After washing with PBS, cells were permeabilized for 10 min with 0.5% Triton X-100 in PBS, washed again, and incubated with anti–myc antibody for 20 min at room temperature. After washing, cells were incubated with anti–mouse antibody conjugated with fluorescein or Texas red for 20 min. After washing three times with PBS, coverslips were mounted on slides using an Anti-Fade kit and cells were photographed using an inverted fluorescence microscope (Eclipse TE200; Nikon Inc.) connected to a digital camera (DKC5000; Sony Corp.).

Incorporation of Bromodeoxyuridine

24 h after transfection, NIH 3T3 cells were serum starved in DMEM supplemented with 2 μg/ml insulin, 2 μg/ml transferrin, and 20 μg/ml BSA, and then stimulated with various agents. After 16 h, cells were incubated for 3 h with bromodeoxyuridine (BrdU, 10 μM), and then fixed in 4% paraformaldehyde containing 5% sucrose, pH 7.0, for 20 min at room temperature. After washing with PBS, cells were incubated in permeabilization buffer (0.5% Triton/PBS, pH 7.4, containing 10 mg/ml BSA) for 20 min at room temperature, and then incubated for 1 h at room temperature with monoclonal anti–BrdU antibody in the presence of DNAse (1,000 U/ml) (Van Brocklyn et al., 1998). After washing with PBS, cells were stained with Texas red–conjugated anti–mouse antibody in 5% BSA/PBS for 1 h, washed with PBS, and then photographed using an inverted fluorescence microscope connected to a digital camera. Cells expressing GFP and cells with positive BrdU staining were counted. At least three different fields were scored with a minimum of 100 cells scored per field.

Measurement of DNA Synthesis

Stably transfected NIH 3T3 fibroblasts were plated in 24-well clusters at a density of 5×10^3 cells/well in DMEM containing 10% calf serum. After 24 h, cells were washed with DMEM containing 0.5% calf serum and incubated in same media. The media was replaced every 2–3 d. At the indicated times, cultures were pulsed with 1 μCi of [^{3}H]thymidine for 6 h and radioactivity incorporated into trichloroacetic acid–insoluble material measured as previously described (Olivera and Spiegel, 1993). Values are the means of triplicate determinations and standard deviations were routinely <10% of the mean.

Cell Cycle Analysis

Stably transfected NIH 3T3 fibroblasts were trypsinized and counted. Aliquots containing 2×10^6 cells were centrifuged, washed twice with PBS, and resuspended in 40 mM citrate buffer, pH 7.6, containing 250 mM sucrose and 5% DMSO. After propidium iodide staining of cellular DNA, cell cycle analysis was performed with a FACStar$^{plus®}$ flow cytofluorometer (Becton Dickinson & Co.) (Goodemote et al., 1995).

Analysis of Cell Growth

Stably transfected NIH 3T3 fibroblasts (1,000 cells) were plated in 24-well plates in DMEM containing 10% calf serum. After 24 h, cells were washed twice with DMEM and then grown in DMEM containing 0.5 or 10% calf serum. At the indicated times, cells were washed with PBS, fixed with 70% ethanol for 10 min, and stained with crystal violet. Incorporated dye was dissolved in 100 μl of 0.1 M sodium citrate in 50% ethanol, pH 4.2, and the absorbance was measured at 540 nm (Wang et al., 1999a). In some experiments, cells were trypsinized and counted in a hemocytometer.

Determination of Apoptotic Cells

Apoptosis was assessed by staining cells with 8 μg/ml Hoechst in 30% glycerol/PBS for 10 min at room temperature as previously described (Cuvillier et al., 1998). Cells expressing GFP were examined with an inverted fluorescence microscope. Apoptotic cells were distinguished by condensed, fragmented nuclear regions. The percentage of intact and apoptotic nuclei in cells expressing GFP fluorescence was determined (Van Brocklyn et al., 1999). A minimum of 500 cells were scored in a double-blinded manner to minimize subjective interpretations. In some experiments, viable cells were determined by trypan blue exclusion.

Fluorogenic DEVD Cleavage Enzyme Assays

Enzyme reactions were performed in 96-well plates with 20 μg of cytosolic proteins and a final concentration of 20 μM Ac-DEVD-AMC substrate as previously described (Cuvillier et al., 1998). Fluorescent AMC product formation was measured over a 30-min period at excitation and emission wavelengths of 360 and 460 nm using a Cytofluor II fluorometer plate reader (PerSeptive Biosystems).

Western Blotting

Cytosolic fractions (10–25 μg) were boiled in Laemmli sample buffer, separated on 10 or 15% SDS-PAGE, and blotted to nitrocellulose. The membranes were blocked with nonfat dry milk in 0.1% Tween-20–PBS for 2 h, and incubated overnight with anti–c-myc monoclonal antibody (1 μg/ml) or 2 h with rabbit antiserum specific for the p17 subunit of caspase-3 (kindly provided by Dr. Donald Nicholson, Merck), in the same buffer containing 1% nonfat dry milk. Immunocomplexes were detected by enhanced chemiluminescence as described previously (Cuvillier et al., 1998).

Results

Characterization of Cells Expressing Sphingosine Kinase

Similar to our previous results with transiently transfected cells (Kohama et al., 1998), sphingosine kinase activity in cell lysates from NIH 3T3 and HEK293 cells stably expressing c-myc–tagged SPHK1a was dramatically increased (500-fold; Fig. 1 A). Western blot analysis of cytosolic fractions using anti–c-myc antibody revealed a specific protein band with an apparent molecular weight consistent with the predicted size of c-myc–sphingosine kinase that was absent in vector-transfected cells (Fig. 1 A, insert). SPP levels were also elevated in cells expressing sphingosine kinase by four- to eightfold (Fig. 1 B), a level that did not correlate with the large fold increase in sphingosine kinase activity measured in vitro. One possible explanation for this discrepancy is that availability of cellular

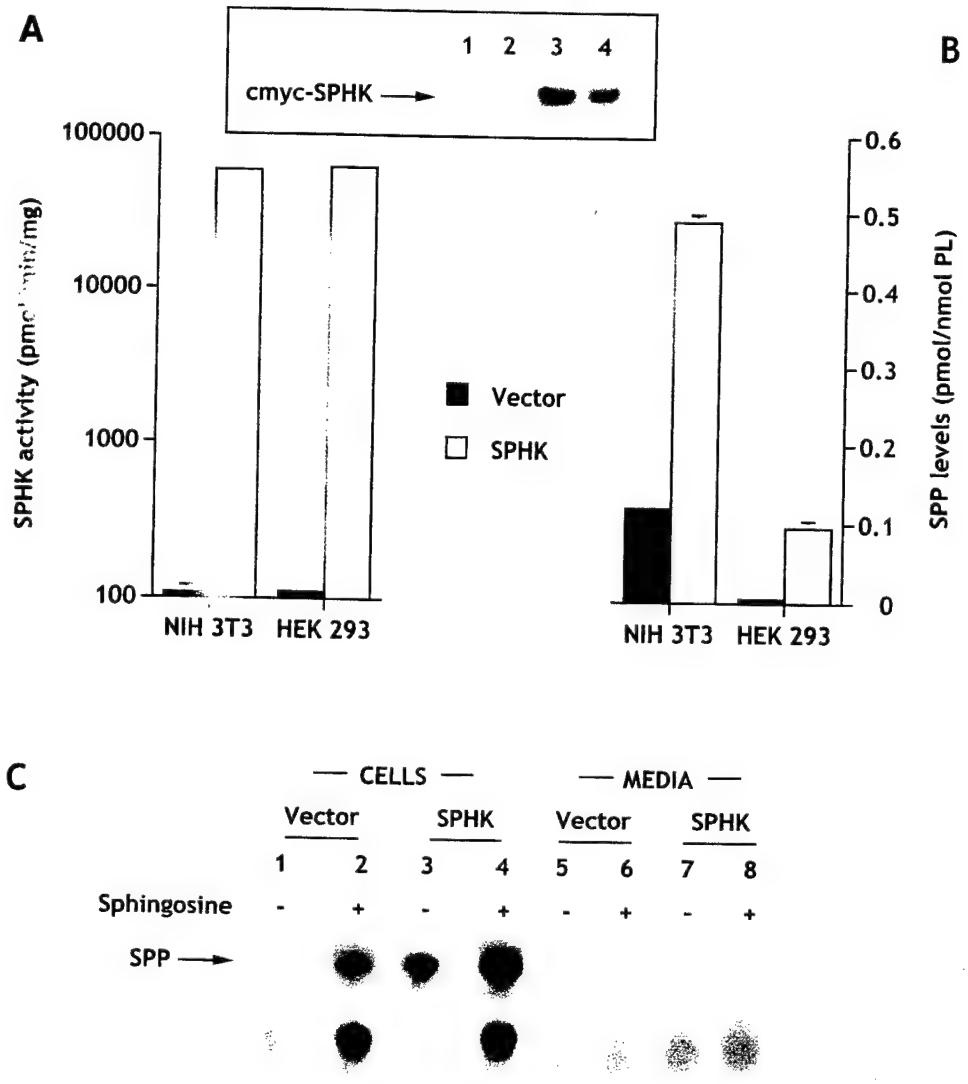


Figure 1. Sphingosine kinase expression results in an increase in intracellular but not extracellular levels of sphingosine-1-phosphate. (A) Cytosolic sphingosine kinase activity was measured in cells stably transfected with c-myc–pcDNA3 (filled bars) or c-myc–pcDNA3-SPHK1a (open bars). The sphingosine kinase activity in vector-transfected cells was 108 ± 12 and 111 ± 7 pmol/min per mg for NIH 3T3 and HEK293 cells, respectively. (Inset) Western blot showing expression of sphingosine kinase. (1 and 2) Vector-transfected HEK293 and NIH 3T3 cells, respectively; (3 and 4) SPHK1a-transfected HEK293 and NIH 3T3 cells, respectively. (B) SPP levels in cells stably expressing sphingosine kinase. Transfected cells were washed and incubated for 24 h in serum-free media (HEK293 cells) or serum-free media containing fatty acid–free BSA (20 μg/ml), transferrin (2 μg/ml), and insulin (2 μg/ml) (NIH 3T3 fibroblasts). SPP was extracted and measured as described in Materials and Methods. Levels of SPP in vector-transfected cells were 0.119 ± 0.003 and 0.012 ± 0.002 pmol/nmol phospholipid for NIH 3T3 and HEK293 cells, respectively. (C) Cellular and secreted [^{32}P]SPP. HEK293 cells labeled to isotopic equilibrium with [^{32}]P_i for 24 h. [^{32}P]SPP was then extracted from the cells and the media as described in Materials and Methods. Duplicate cultures were treated with 5 μM sphingosine 10 min before extraction of lipids (lanes 2, 4, 6, and 8). In the autoradiograms of the TLC analyses, the arrow indicates the location of SPP visualized with molybdenum blue spray.

sphingosine might limit the production of SPP. In agreement with our previous results (Zhang et al., 1991), when cells were acutely treated with exogenous sphingosine, which is readily taken up (Olivera et al., 1994, 1997), levels of SPP were further increased three- to sixfold, suggesting that although availability of sphingosine may be important for regulating SPP levels, it is probably not the only critical factor influencing levels of SPP in cells overexpressing sphingosine kinase.

Since activated platelets can release SPP (Yatomi et al., 1995), and the EDG family of G protein–coupled SPP receptors have recently been identified (An et al., 1997; Goetzl and An, 1998; Lee et al., 1998; Okamoto et al., 1998; Van Brocklyn et al., 1998, 1999; Zondag et al., 1998; Gonda et al., 1999), it was important to determine whether sphingosine kinase–transfected cells, which have notable increases in SPP levels, secrete SPP into the medium. No significant release of SPP into the extracellular media could be detected by our mass measurements, even after addition of sphingosine to both NIH and HEK293 sphingosine kinase–transfected cells. To increase the sensitivity of detection of secreted SPP, we labeled HEK 293 cells to isotopic equilibrium with [^{32}P]P_i and analyzed the labeled SPP in cells as well as in the medium (Fig. 1 C). Despite the large increases in [^{32}P]SPP detected in cells overexpressing sphingosine kinase, there was no detectable labeled SPP released into the medium. Similar results were also found with NIH3T3 fibroblasts. However, in agreement with previous studies (Yatomi et al., 1997a), we could readily detect secretion by human platelets after thrombin treatment. Both methods to measure SPP levels gave identical increases in intracellular SPP in transfected and sphingosine-treated cells. Based on the sensitivity of these methods (1–2 pmol SPP/sample), it is estimated that the concentration of SPP in the extracellular media must be <0.4 nM, a concentration well below the K_d for binding

Table I. PDGF Stimulates Sphingosine Kinase Activity in Sphingosine Kinase–transfected Cells

Treatment	Cytosolic sphingosine kinase activity		Cytosolic sphingosine kinase activity	
	Vector	SPHK		
	percent of total activity		*pmol/min/mg*	
None	69	68	68	5,900
PDGF (1 ng/ml)	ND	ND	86	8,992
PDGF (20 ng/ml)	71	78	124	9,092

NIH 3T3 cells transfected with c-myc–pcDNA3 (Vector) or c-myc–pcDNA3-SPHK1a (SPHK) were incubated in serum-free media containing fatty acid–free BSA (20 μg/ml), and transferrin (2 μg/ml) overnight, and then stimulated with the indicated concentrations of PDGF for 10 min. Sphingosine kinase activity in cytosol and particulate fractions was measured as described in Materials and Methods and standard deviations were <10%.

of SPP to its receptors (8–25 nM) (Van Brocklyn et al., 1998, 1999).

Localization of Sphingosine Kinase and Stimulation by Growth Factors

Both membrane-associated and cytosolic sphingosine kinase activities have been described in mammalian tissues and cell lines (Stoffel et al., 1973; Buehrer and Bell, 1992; Olivera and Spiegel, 1993; Ghosh et al., 1994; Olivera et al., 1994). However, the amino acid sequence of murine SPHK1a suggests that it should be a cytosolic protein (Kohama et al., 1998). In agreement with our previous study on transiently transfected cells (Kohama et al., 1998), most of the sphingosine kinase activity in cells stably expressing c-myc–tagged sphingosine kinase was cytosolic and the relative amounts of sphingosine kinase activity in cytoplasmic versus membrane fractions were similar in vector- and sphingosine kinase–transfected cells (Table I), suggesting that the small c-myc tag does not affect localization of sphingosine kinase. Immunohistochemistry with antibodies against c-myc revealed that sphingosine kinase has a diffuse distribution in the cytosol and somewhat denser expression in perinuclear sites in both NIH 3T3 and HEK 293 cells (Fig. 2).

Previously, we and others have shown that PDGF stimulates sphingosine kinase activity in various cell types (Olivera and Spiegel, 1993; Bornfeldt et al., 1995; Pyne et al., 1996; Rani et al., 1997). PDGF stimulated cytosolic c-myc–sphingosine kinase activity in transfected NIH 3T3 fibroblasts to a similar extent as its effect on endogenous sphingosine kinase (Table I), indicating that c-myc–sphingosine kinase activity is regulated by the signaling pathways triggered by growth factors in the same manner as the native enzyme. Unlike activation of protein kinase C, activation of sphingosine kinase by PDGF does not induce significant translocation to the membrane fraction. Collectively, these data suggest that cells overexpressing sphingosine kinase are a useful tool to study intracellular actions of SPP. Interestingly, endogenous SPP was present in both the cytoplasm and membrane fractions and overexpression of sphingosine kinase markedly increased both, by nearly the same extent (Table II). In contrast to most phospholipids, glycosphingolipids, and other sphingolipid metabolites, such as ceramide and sphingosine, which are found mainly in membranes, 20% of the total cellular SPP is in the cytosol (Table II).

Effect of Sphingosine Kinase Overexpression on Cell Proliferation

Studies with sphingosine kinase inhibitors suggested that the mitogenic effect of SPP might be due to intracellular actions (Olivera and Spiegel, 1993; Van Brocklyn et al., 1998). However, others have suggested that the mitogenic effect is mediated by binding of SPP to the EDG family of G protein–coupled receptors (Goetzl and An, 1998). In view of this controversy, it was of interest to examine the proliferation of cells whose intra- rather than extracellular levels of SPP are increased after transfection with sphingosine kinase. Transient expression of c-myc–sphingosine-1-phosphate (SPHK) in NIH 3T3 cells not only increased intracellular levels of SPP, it also increased the proportion of cells in the S phase of the cell cycle measured as incorporation of BrdU into nascent DNA (Fig. 3 A). The growth promoting effects of sphingosine kinase were further enhanced by suboptimal concentrations of PDGF (1 ng/ml) and serum (0.1%) (Fig. 3 A), which are known to stimulate sphingosine kinase (Olivera and Spiegel, 1993, and Table I). However, addition of exogenous SPP increased BrdU incorporation in empty vector-transfected cells twofold, but had no effect on sphingosine kinase–transfected cells, suggesting that these cells are already maximally stimulated by their intracellular SPP. It should be pointed out that these experiments were carried out in the presence of 2 μg/ml insulin, which is a known survival factor for these cells and was used to prevent cell death caused by serum deprivation.

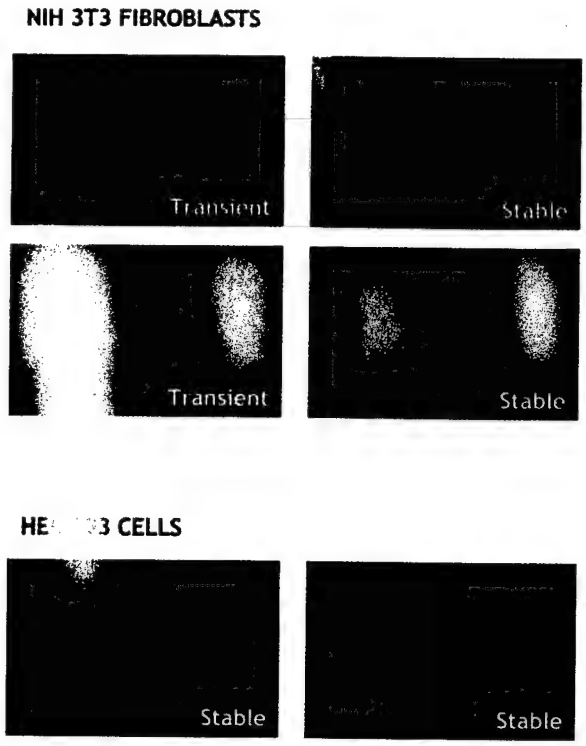
Since transient expression of sphingosine kinase increased the proportion of cells in the S phase, it was of interest to determine whether there was a corresponding increase as a result of stable transfection. Nonclonal pools of stably transfected cells were studied to avoid potential phenotypic changes due to selection and propagation of clones derived from single individual cells. Stable expression of sphingosine kinase had a dramatic effect on [^{3}H]thymidine incorporation into DNA in cells cultured in low serum media, while DNA synthesis was low in empty vector-transfected cells over the course of 8 d in 0.5% serum (Fig. 4 A).

DNA flow cytometry was used to characterize the distri-

Table II. Distribution of SPP in Membrane and Cytosol Fractions

Fraction	SPP		SPP	
	Vector	SPHK	Vector	SPHK
	pmol/mg protein		*pmol/nmol phospholipid*	
Cytosol	0.45 ± 0.014	2.17 ± 0.012	0.015 ± 0.003	0.111 ± 0.044
Membrane	1.7 ± 0.16	9.1 ± 0.37	0.009 ± 0.003	0.056 ± 0.007

SPP in cytosol and particulate fractions of HEK 293 cells transfected with c-myc–pcDNA3 (Vector) or c-myc–pcDNA3-SPHK1a (SPHK) was measured as described in Materials and Methods. The same samples were also analyzed for phospholipid content.



bution of cells in the cell cycle. FACS® analysis revealed that after 2 d in 0.5% serum, >95% of the vector-transfected NIH 3T3 fibroblasts were in G_0/G_1 phase and only a small fraction were in S and G_2/M phases (Table III). Overexpression of sphingosine kinase reduced the fraction of cells in G_0/G_1 and, in agreement with the DNA synthesis data, increased the proportion in the S phase and, to a lesser extent, in the G_2/M phase. Even in the presence of 10% serum, which markedly increased the proportion of cells in the S phase and G_2/M phase, sphingosine kinase transfection further increased the proportion of cells in the S phase (Table III). This data suggests that either a greater proportion of sphingosine kinase–transfected cells are cy-

Figure 2. Cellular localization of c-myc–sphingosine kinase. NIH3T3 fibroblasts transiently or stably expressing sphingosine kinase and HEK293 cells stably expressing sphingosine kinase were incubated with a monoclonal c-myc antibody (20 μg/ml) and stained with anti–mouse Texas red–conjugated IgG (1:100 dilution) (left) or anti–mouse FITC-monoclonal IgG (1:10 dilution) (right). Fluorescence micrographs (60×) were taken using an inverted fluorescent microscope. Vector transfectants (c-myc–pcDNA3) did not show any significant fluorescence.

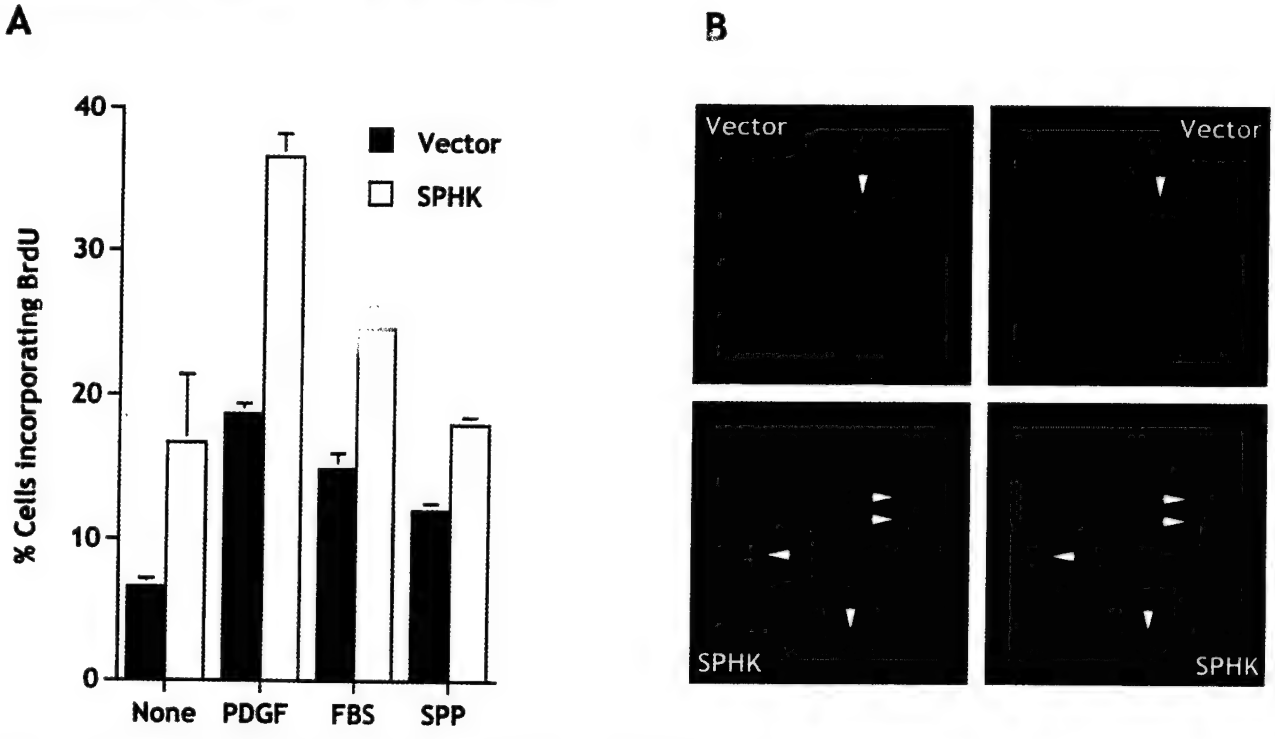


Figure 3. Expression of sphingosine kinase stimulates BrdU incorporation into nascent DNA. (A) NIH 3T3 fibroblasts were transiently transfected with empty vector (pCMV-SPORT2, filled bars) or pCMV-SPORT2-SPHK1a (open bars) together with pCEFL-GFP. Cells were serum-starved for 18 h and incubated in serum-free media supplemented with insulin (2 μg/ml) without (None) or with PDGF (1 ng/ml), FBS (0.1%), or SPP (10 μM). After 16 h, BrdU was added for an additional 3 h. Double immunofluorescence was used to visualize transfected cells and BrdU incorporation, and the proportion of cells incorporating BrdU among total transfected cells (expressing GFP) was determined. Data are means ± SD of duplicate cultures from a representative experiment. At least three different fields were scored with a minimum of 100 cells scored per field. Similar results were obtained in three independent experiments. For comparison, in medium containing 10% FBS, 42.3 ± 4.9% of the vector-transfected cells incorporated BrdU. (B) Representative images of cells treated with a suboptimum concentration of PDGF (1 ng/ml). Vector- and sphingosine kinase–transfected NIH 3T3 cells expressing GFP (left) and incorporating BrdU (right) were visualized by double immunofluorescence. Arrows indicate cells that are positive for both.

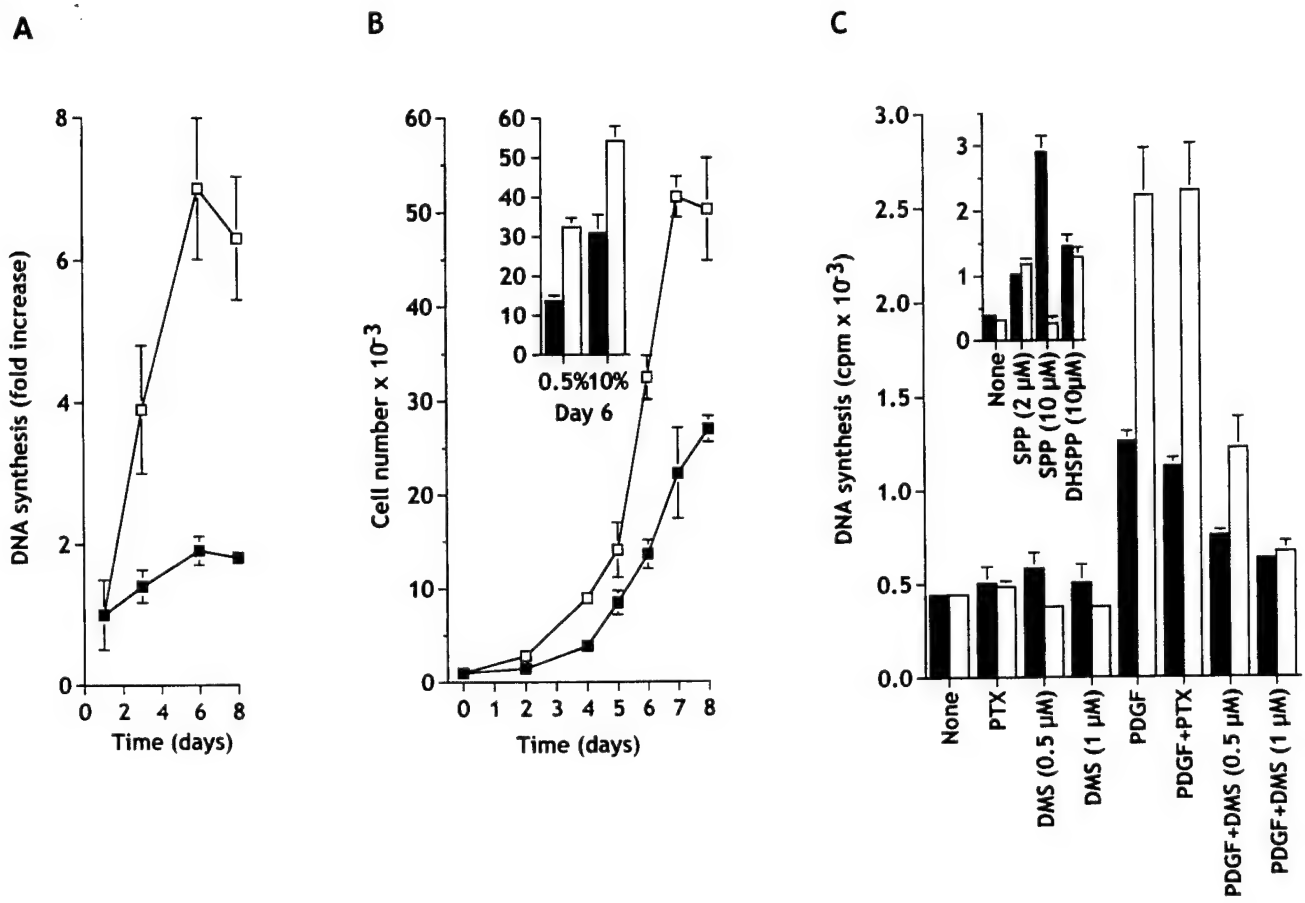


Figure 4. Expression of sphingosine kinase stimulates growth of NIH 3T3 fibroblasts cultured in low serum. (A) Cells stably transfected with c-myc–pcDNA3 (■) or with c-myc–SPHK-pcDNA3 (□) were plated at low density, washed after 24 h, and cultured in 0.5% calf serum for the indicated days. Media was replaced every 2 d, the cells were pulsed at the indicated times with [^{3}H]thymidine for an additional 6 h, and DNA synthesis measured as described in *Materials and Methods*. Data are means ± SD of three independent determinations and are expressed as fold increase of the value determined after 1 d. Similar results were obtained in three additional experiments. (B) Sphingosine kinase–transfected NIH 3T3 fibroblasts proliferate more rapidly than control cells. Cells stably transfected with c-myc–pcDNA3 (■) or with c-myc–SPHK-pcDNA3 (□) were plated in 24-well tissue culture plates (1,000 per well), cultured for the indicated days in the presence of 0.5% CS (B) or for 6 d in 10% CS (insert), stained with crystal violet, and quantitated as described in *Materials and Methods*. Media was replaced every 2 d. Similar results were obtained in at least two additional experiments. (C) *N,N*-Dimethylsphingosine, but not pertussis toxin, inhibits DNA synthesis induced by sphingosine kinase overexpression. Cells stably transfected with c-myc–pcDNA3 (filled bars) or with c-myc–SPHK-pcDNA3 (open bars) were plated at 2×10^4 cells per well, washed after 24 h, and cultured in serum-free DMEM containing 2 μg/ml transferrin and 20 μg/ml BSA, in the absence or presence of the indicated agents. After 16 h, cells were pulsed with 1.0 μCi of [^{3}H]thymidine for 8 h, and incorporation of [^{3}H]thymidine into trichloroacetic acid–insoluble material was measured. Values are the means ± SD of triplicate determinations, and similar results were found in three independent experiments. Agents (concentration): pertussis toxin (PTX; 50 ng/ml); DMS (0.5 or 1 μM); PDGF (20 ng/ml); SPP (2 or 10 μM); dihydro-SPP (DHSPP, 10 μM).

cling and/or that the duration of the G_1 phase is shortened compared with vector-transfected cells.

Growth curves in low serum of vector and sphingosine kinase–transfected 3T3 fibroblasts diverged after 2 d and growth of the sphingosine kinase–transfected cells was markedly enhanced thereafter, demonstrating that the alteration in cell-cycle distribution resulted in increased growth rate (Fig. 4 B). Moreover, even in the presence of 10% serum, sphingosine kinase expression was still able to significantly increase proliferation (Fig. 4 C), and to increase the saturation density (not shown).

Analyses of the growth curves during the exponential growth phase revealed that sphingosine kinase expression decreased the doubling time in 0.5% calf serum from 34 ± 1.4 to 27 ± 1.8 h, although no differences were observed in the doubling times in full serum (vector, 17 ± 2.2 h; SPHK, 16 ± 1.2 h). Furthermore, computation of the parameters of the cell cycle during exponential growth revealed that sphingosine kinase markedly shortened the duration of G_1 phase of the cell cycle by 31 ± 2% in low serum, suggesting that sphingosine kinase is important for nontransformed cells to progress through the G_1-S boundary. However, in cells growing in full serum, although overexpression of sphingosine kinase increases the proportion of cells in S phase with a corresponding decrease in G_0/G_1 (Table III), there were no significant changes in the duration of either of these phases of the cell cycle.

To further investigate the possibility that the EDG fam-

Table III. Flow Cytometric Analysis of Cell

Cells	Cell cycle distribution		
	G_0/G_1	S	G_2/M
	Percentage of cells		
Vector, 0.5% CS	96.4 ± 0.8	1.6 ± 0.4	1.9 ± 0.4
SPHK, 0.5% CS	90.4 ± 0.7*	6.4 ± 0.1*	3.2 ± 0.4*
Vector, 10% CS	65.4 ± 0.1*	25.4 ± 0.2*	9.5 ± 0.2*
SPHK, 10% CS	57.5 ± 0.7*	32.1 ± 0.6*	10.4 ± 0.1‡

NIH 3T3 cells stably transfected with c-myc–pcDNA3 (Vector) or c-myc–pcDNA3-SPHK1a (SPHK) were plated at 500,000 cells/150-mm dish. After 24 h, cells were washed twice with DMEM and incubated in media containing 0.5 or 10% CS for 2 d. Cell-cycle analysis was then performed by flow cytometry as described in Materials and Methods.

*Significant differences from vector-transfected values as determined by Student's *t* test ($P \leq 0.01$).

‡Not significantly different.

ily of SPP receptors might be involved in the proliferative response induced by overexpression of sphingosine kinase, stably transfected 3T3 fibroblasts were treated with pertussis toxin, as these receptors are known to act through pertussis toxin-sensitive G proteins (Lee et al., 1998; Van Brocklyn et al., 1998; Kon et al., 1999; Sato et al., 1999). In contrast to many other biological responses, including the mitogenic effect of exogenous SPP, which are inhibited by pertussis toxin (Goodemote et al., 1995), it did not abrogate the increase in DNA synthesis induced by expression of sphingosine kinase in the presence of PDGF, suggesting that sphingosine kinase acts independently of G_i proteins (Fig. 4 C). In contrast, treatment of cells with a specific inhibitor of sphingosine kinase, *N,N*-dimethylsphingosine (DMS), at a concentration that inhibits sphingosine kinase and decreases SPP levels, blocked the effects of sphingosine kinase overexpression (Fig. 4 C). Similar results were obtained in cells transiently expressing sphingosine kinase (data not shown). Moreover, although 2 μM SPP stimulated proliferation in vector and sphingosine kinase–transfected cells (Fig. 4 C, inset), 10 μM SPP markedly stimulated DNA synthesis of vector-transfected cells while inhibiting proliferation of sphingosine kinase–transfected cells. Interestingly, 10 μM dihydro-SPP, which lacks the trans double bond present in SPP and binds and signals through Edg-1, -3, and -5 (Van Brocklyn et al., 1998, 1999), only slightly stimulated DNA synthesis in both vector- and kinase-transfected cells (Fig. 4 D, inset), suggest-

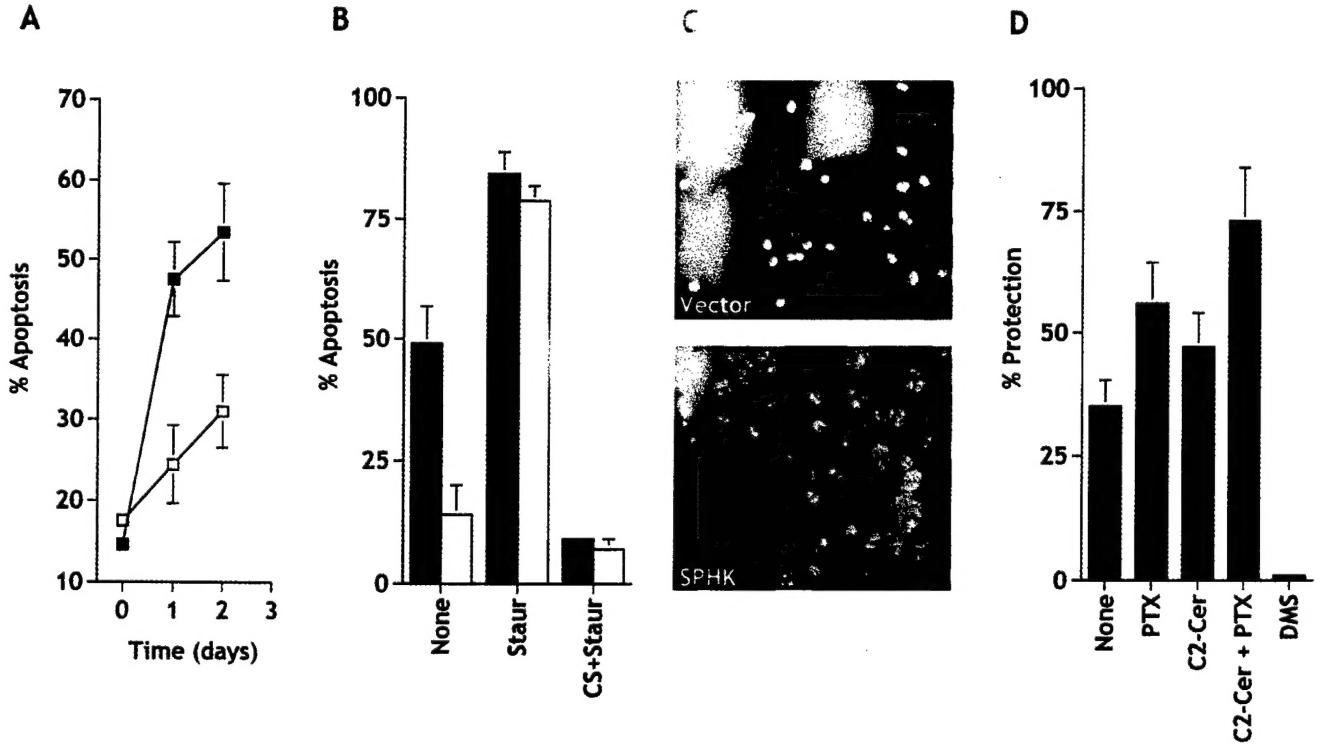


Figure 5. Expression of sphingosine kinase in NIH 3T3 fibroblasts reduces apoptosis induced by serum deprivation. (A) NIH 3T3 fibroblasts were transiently transfected with vector (■) or SPHK1a (□), together with pCEFL-GFP, and serum starved for the indicated times. Total GFP-expressing cells and GFP-expressing cells displaying fragmented nuclei indicative of apoptosis were counted as described in *Materials and Methods*. A minimum of 500 cells in each field were scored. Data are mean ± SEM of three independent experiments, each one done in duplicate or triplicate. (B) Stably vector-transfected (filled bars) or c-myc–tagged SPHK1a (open bars)-transfected NIH 3T3 cells were serum starved in the absence (None) or presence of 50 nM staurosporine (Staur) or 10% serum plus 250 nM staurosporine (CS+Staur) for 24 h. Percentages of apoptotic cells were determined after Hoechst staining by fluorescence microscopy. (C) Note the typical condensed fragmented nuclei of apoptotic cells in vector but not in SPHK-overexpressing cells after serum deprivation. (D) DMS, but not pertussis toxin, inhibits the protective effect of sphingosine kinase. Stably vector- or c-myc–tagged SPHK1a-transfected NIH 3T3 cells were serum starved in the absence (None) or presence of 5 μM DMS, 20 ng/ml pertussis toxin (PTX), 25 μM C2-ceramide (C2-Cer) or both for 24 h, and the number of viable cells was determined as described in *Materials and Methods*. Percent protection from apoptosis by sphingosine kinase expression = 100× [(percent viable sphingosine kinase cells − percent of viable vector cells)/(percent of viable vector-transfected cells)].

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Effect of Sphingosine Kinase Overexpression on Programmed Cell Death

Exogenous SPP has been shown to suppress apoptosis induced by cytokines, such as TNF and Fas ligand (Cuvillier et al., 1996), serum deprivation (Edsall et al., 1997; Van Brocklyn et al., 1998), and to be important in the survival effects of NGF (Edsall et al., 1997), vitamin D3 (Kleuser et al., 1998), and cAMP (Machwate et al., 1998). In contrast, more recently it has been suggested that SPP protected human T lymphoblastoma cells from apoptosis induced by antibodies to Fas, CD2, and CD3/CD28, or C6-ceramide by binding to EDG-3 and EDG-5 GPCRs (Goetzl et al., 1999). However, if the intracellular level of SPP is a critical factor that determines cell survival, then it is expected that overexpression of sphingosine kinase should suppress apoptosis. In agreement with previous studies (Brancolini et al., 1997), prolonged serum deprivation induced apoptosis in NIH 3T3 fibroblasts (Fig. 5 A), where shrinkage and condensation of nuclei were clearly evident (Fig. 5 C). Transient and stable expression of sphingosine kinase in NIH 3T3 fibroblasts suppressed the appearance of apoptotic nuclei induced by serum starvation (Fig. 5, A–C). In contrast, sphingosine kinase expression had almost no effect on apoptosis resulting from treatment with staurosporine (Fig. 5 B), a broad spectrum protein kinase inhibitor that is known to induce apoptosis in normal and neoplastic cells (Jacobsen et al., 1996). Moreover, DMS, but not pertussis toxin, inhibited the cytoprotective effect of sphingosine kinase overexpression in stably (Fig. 5 D) as well as transiently transfected NIH 3T3 fibroblasts.

It should be pointed out that serum withdrawal markedly increases ceramide levels in many cell types, and it has been proposed that ceramide mediates, at least in part, serum deprivation–induced cell death (Hannun, 1996). Thus, it was of interest to examine the effect of sphingosine kinase expression on apoptosis of other types of cells. Expression of sphingosine kinase in HEK293 cells also markedly inhibited apoptosis induced by serum deprivation and by the cell permeable ceramide analogue, C2-ceramide, although to a lesser extent (Fig. 6 A). These cytoprotective effects of sphingosine kinase overexpression were also not inhibited by pertussis toxin treatment. In contrast, apoptosis induced by staurosporine, a well recognized inducer of apoptosis in HEK293 cells (Ozawa et al., 1999), was not suppressed by expression of sphingosine kinase (Fig. 6 A).

Caspases, a family of aspartate-specific cysteine proteases, play a critical role in the execution phase of apoptotic cell death by cleavage of a specific set of cytosolic and nuclear proteins leading to disassembly of the cell (Nicholson et al., 1995; Thornberry and Lazebnik, 1998). Fig. 6 B demonstrates that serum withdrawal induced activation of caspase-3, as shown by the appearance of the p20 and p17 subunits, which correlated with the onset of apoptosis. Overexpression of sphingosine kinase reduced processing of caspase-3 (Fig. 6 B).

Human leukemic Jurkat T cells are a well-characterized model system that readily undergo apoptosis and are sensitive to C2-ceramide–induced apoptosis (Cu
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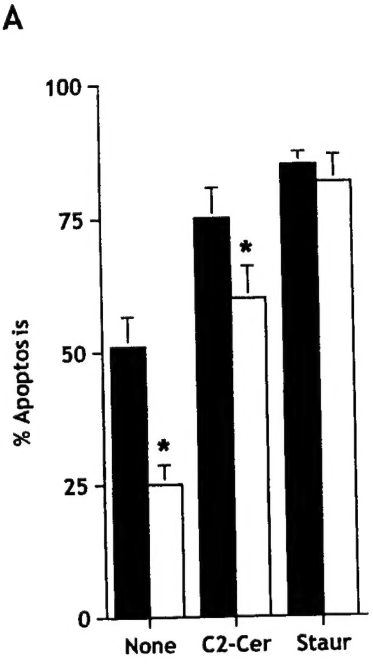

Figure 6. Sphingosine kinase inhibits serum-depri
C2-ceramide–induced apoptosis, but not staurospo
apoptosis, in HEK293 cells. (A) HEK293 cells stabl
empty vector (filled bars) or c-myc–tagged SPHK1a
were incubated in serum-free medium for 30 h, and
in the absence (None) or presence of 25 μM C2-ce
Cer) or 100 nM staurosporine (Staur) for 24 h. Pe
apoptotic cells were determined after Hoechst stai
rescence microscopy. Data are means ± SEM of f
dent experiments, each done in triplicate. *Significan
from vector-transfected values ($P \leq 0.05$). (B) E
sphingosine kinase inhibits activation of caspase-3. F
expressing empty vector (Vect) or c-myc–tagg
(SPHK) were incubated in serum-free medium and
tracts were prepared at the indicated times. Prote
solved by 15% SDS-PAGE, blotted, and probe
caspase-3. Migrations indicated are: full-length c
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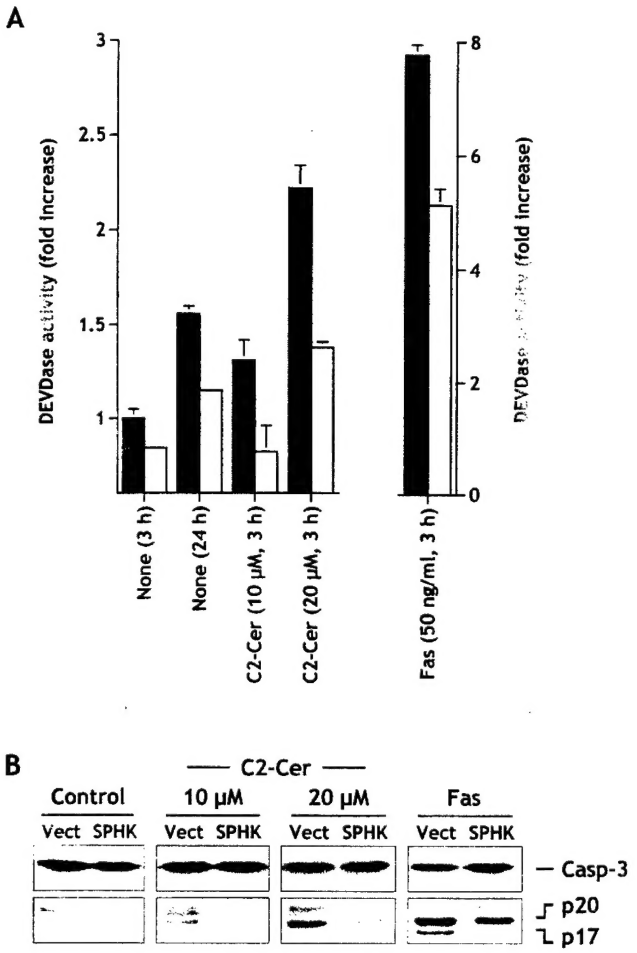

Figure 7. Expression of sphingosine kinase suppresses DEVDase activity and inhibits caspase-3 activation induced by serum starvation. C2-ceramide and Fas ligation in Jurkat T cells. (A) Jurkat T cells transfected with vector (filled bars) or SPHK1a (open bars) were serum starved for 3 or 24 h or incubated for 3 h in serum-free media in the presence of 10 or 20 μM C2-ceramide (C2-Cer) or agonistic Fas antibody (50 ng/ml). Activation of DEVD-specific caspases was measured by the cleavage of the fluorogenic substrate Ac-DEVD-AMC. All values for sphingosine kinase–transfected cells were significantly different from vector-transfected cells as determined by Student's *t* test ($P \leq 0.01$). (B) Cellular extracts from Jurkat cells stably transfected with empty vector (Vect) or SPHK1a expression vector (SPHK) were treated for 3 h without (Control) or with the indicated concentration of C2-ceramide or anti–Fas as described in A, separated by SDS-PAGE and immunoblotted with anti–caspase-3. The migration positions of full-length 32-kD caspase-3 and proteolytically processed intermediate p20 and active subunit p17 are indicated.

pression of sphingosine kinase (data not shown). The fluorogenic substrate Ac-DEVD-AMC, which corresponds to the cleavage site found in executioner caspases targets, was used to measure the activation of these caspases. Indeed, serum deprivation, Fas ligation, and C2-ceramide treatment of Jurkat cells resulted in a time-dependent increase in DEVDase proteolytic activity (Fig. 7 A), which preceded the appearance of fragmented nuclei. Overexpression of sphingosine kinase blocked the increase in DEVDase activity induced by serum deprivation and C2-ceramide (Fig. 7 A) and had a lesser yet still significant effect on Fas ligation–induced DEVDase activation. In agreement, sphingosine kinase effectively prevented activation of caspase-3, as measured by the decrease of its processing to p20 and p17 forms following serum deprivation and C2-ceramide addition, but had a smaller effect on Fas-induced activation (Fig. 7 B).

Discussion

Apparently contradictory reports describe intra- and extracellular actions of SPP in diverse cell types (Goetzl and An, 1998; Spiegel, 1999). While much evidence has accumulated recently indicating that SPP functions as a ligand for the EDG family of GPCRs, which are linked to diverse biological responses (Goetzl and An, 1998; Spiegel, 1999), less is known of the role of SPP as a second messenger. SPP has been proposed as an intracellular mediator of cell growth (Spiegel et al., 1996), mainly based on the use of exogenously added SPP in the high micromolar range, stimulation of sphingosine kinase by growth factors, as well as sphingosine kinase inhibitors. For example, in Swiss 3T3 cells, competitive inhibitors of sphingosine kinase block some of the proliferative signals elicited by PDGF (Olivera and Spiegel, 1993), including activation of mitogen-activated protein kinase, cyclin-dependent kinases (Cdc2 and Cdk2 kinases), and activation of the transcription factor AP1 (Rani et al., 1997; Su et al., 1994). However, the proliferative response to exogenously added SPP is partially sensitive to pertussis toxin, suggesting the potential involvement of G_i-coupled cell surface receptors (Goodemote et al., 1995). Thus, it was important to develop another approach to unequivocally determine the role of intracellularly generated SPP. To this end, we transiently and stably transfected cells with sphingosine kinase, which markedly increased intracellular levels of SPP. In contrast to activated platelets that release stored SPP in a PKC-dependent manner (Yatomi et al., 1997b), transfected NIH 3T3 fibroblasts and HEK293 cells did not secrete any detectable SPP, suggesting that only certain cell types are capable of secreting SPP and that these transfected cells should be useful to study the intracellular roles of SPP. Interestingly, SPP levels were increased to the same extent by sphingosine kinase expression in both the cytosol and in membrane fractions, whereas other sphingolipid metabolites, ceramide and sphingosine, are predominantly located in membranes. This observation is very intriguing since it might suggest that, similar to another second messenger, inositol trisphosphate, it can move between intracellular compartments.

BrdU incorporation, cell cycle analysis, and growth curves indicate that sphingosine kinase transfection can significantly increase the proliferative rate of nontransformed NIH 3T3 fibroblasts. Enforced expression of sphingosine kinase not only resulted in higher levels of SPP, it also increased the proportion of cells in the S phase of the cell cycle, expedited the G_1/S transition, and reduced the doubling time, especially in low serum conditions, indicating that intracellular SPP is an important regulator of cell growth. In support of this notion, microinjection of SPP (Van Brocklyn et al., 1998) or intracellular generation of SPP by photolysis of incorporated caged SPP (Qiao et al.,

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